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THE UNIVERSITY OF ALBERTA

"PROBLEMS IN THE CONTROL OF BACTERIA
IN OIL-WATER COMBINATIONS"

BY

GERRIE A. LESLIE

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES
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FACULTY OF GRADUATE STUDIES

The undersigned certify that they have read,
and recommend to the Faculty of Graduate Studies for
acceptance, a thesis entitled, "Problems in the
Control of Bacteria in Oil-Water Combinations",
submitted by Gerrie A. Leslie, B.Sc., in partial
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ABSTRACT

The object of this investigation was to study certain problems associated with the control of the growth of bacteria in oil and water combinations through the use of disinfectants.

Various techniques for enumerating and detecting the presence of viable bacteria suspended in oil were studied.

A spectrophotometric procedure was developed to investigate the partitioning effect of phenolic disinfectants between oil and water phases. The effect of time, temperature and hydrocarbon concentration on partitioning was studied. The effect of varying concentrations of phenolic disinfectants, light liquid petrolatum and water on bacterial growth was demonstrated.

Initially the test organisms employed were Staphylococcus aureus F.D.A. 209, Pseudomonas fluorescens O.A.C. 99, and Pseudomonas fluorescens O.A.C. 100. Subsequently Pseudomonas fluorescens O.A.C. 99 was employed to compare lipid and amino acid composition of this organism when grown in a complex medium such as trypticase soy and when grown in a mineral salts medium with light liquid petrolatum as the only carbon and energy source.

Finally the morphological, physiological and some biochemical properties of a new species of Arthrobacter which has the ability to assimilate light liquid petrolatum and use, at least certain components of oil, as a carbon substrate, were studied.

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INTRODUCTION

That certain microorganisms can utilize hydrocarbons as an energy source has been known for many years, though widespread interest in this phenomenon is rather recent.

Microorganisms which can attack hydrocarbons are quite ubiquitous but most frequently found in the soil.

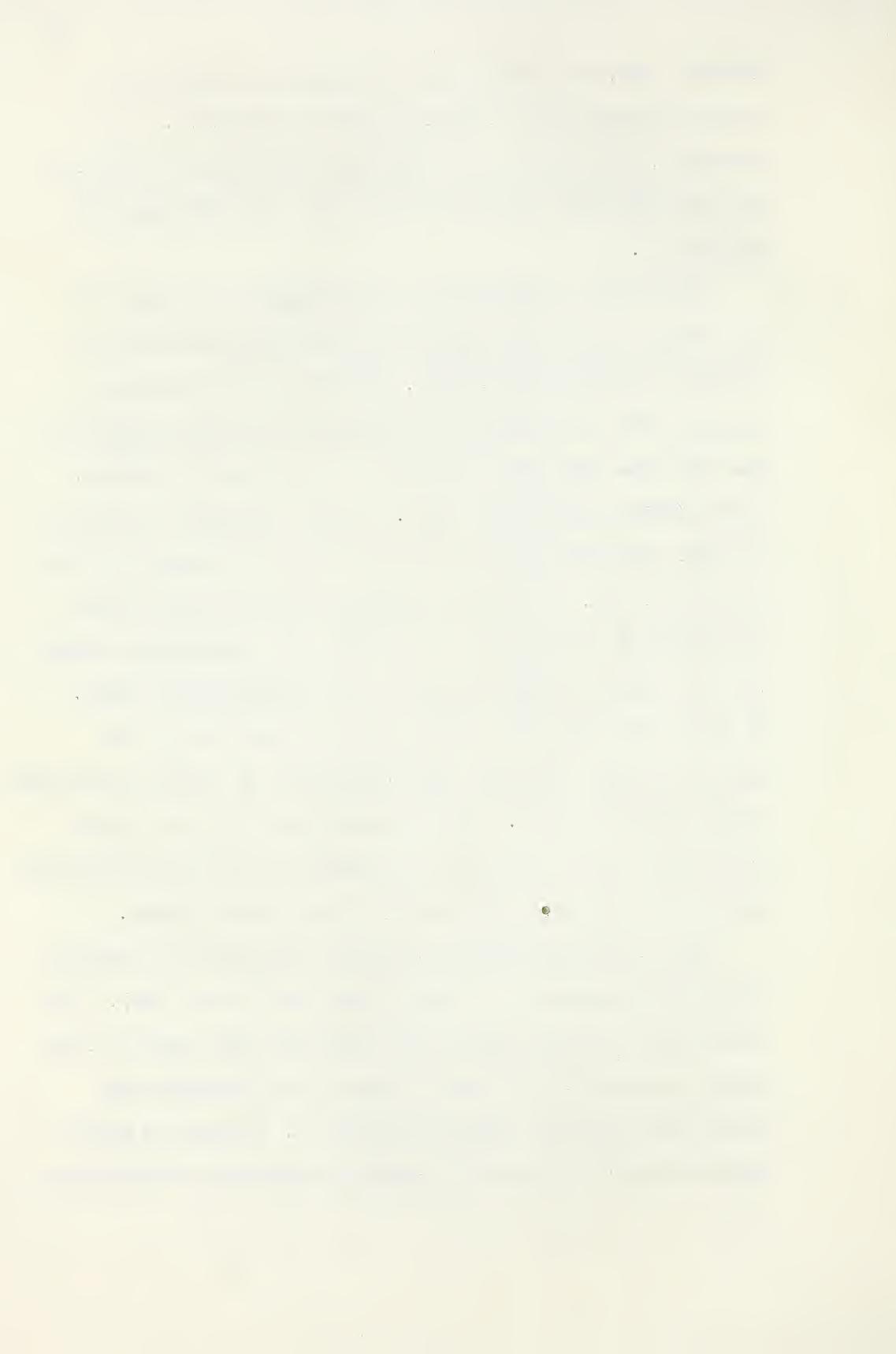
Besides a large number of pure hydrocarbons which have been investigated such as the straight chain alkanes n-hexadecane, n-octadecane, n-hexane, n-tridecane (Raymond and Davis, 1960), n-octane (Baptist, Gholson and Coon, 1963), oxidation of cyclic hydrocarbons by microbes has received considerable attention. Strawinski and Stone (1955) have shown the utilization of naphthalene by a Pseudomonas closely resembling Pseudomonas aeruginosa. Webley et al. (1956) reported high yields of phenylacetic acid from phenyldecanoate, phenylacetate and phenyloctadecane. Davis and Raymond (1961) have shown that Nocardia 107-332, a soil isolate, oxidizes short-chain alkyl-substituted cyclic hydrocarbons to cyclic acids while growing on n-alkanes. Cyclic acids are produced also from relatively long-chain alkyl-substituted cyclics such as n-nonylbenzene or n-dodecylbenzene (Davis and Raymond, 1961).

It is not only pure hydrocarbons which are attacked by microorganisms but many complex compounds such as crude oils, jet and turbine fuels (Kereluk, 1962; Prince, 1961; Kereluk and Baxter, 1963), lubricating oils (Bennett, 1962; Pivnick and Fotopoulos, 1957), tars and asphalt (Traxler, 1962; Phillips and Traxler, 1963;

Traxler, 1963) and most anything of hydrocarbon composition is subject to some degree of attack by some microorganism. It is because of this wide array of substrates that microbial forms can use, that problems arise requiring the use of disinfectants and antiseptics.

Since water is essential for all biological activities, we can assume that microbial activity in petroleum products must be limited by availability of water. Activity for most practical purposes, will be negligible in an aqueous free system although it has been shown that life is possible in an essentially anhydrous medium (DeGray and Killian, 1962). Systems containing a water-oil interface reputedly support microbial activity in proportion to the interfacial area. This is not intended to infer that all viable microorganisms are found at the interface, for considerable numbers have been found at various distances into the hydrocarbon phase. We assume that the major activity is at, or very near to, the hydrocarbon-water interface, and that activity is roughly proportional to the interfacial area. The interfacial area in a static vessel is much less than it would be in an emulsion and this area would vary greatly as to the type and stability of the emulsion system.

Loss of hydrocarbon due to microbial utilization of compounds is probably negligible in a large storage tank (Stone, 1963). This however does not deter from the fact that even small amounts of the product resulting from microbial attack on the hydrocarbon may cause severe secondary consequences later on. Tausen and Shapiro reported in 1934 that, microbial activity and product deterioration



were greatly reduced when the thickness of the hydrocarbon layer was increased. This was probably due to the fact that oxygen is less available at the interface or in the aqueous phase beneath the thick layer of petroleum product and since the majority of microbial processes which result in product utilization are oxidative, this seems to be a reasonable explanation (Evans, 1963).

In spite of the fact that a vast amount of information has been made available concerning the microbial deterioration of hydrocarbon products, very little work has been published dealing with the prevention of this problem.

Oil and water systems are found in the paint industry, soap industry, industries which use cutting fluids as coolants, food processing industries, the cosmetic industry and the pharmaceutical industry. These are but a few examples of where preservation of oil-water systems is a must if loss of time and materials is to be avoided. Probably one of the most pressing needs for preservation is in the aviation industry where the microbial formation of sludges has and can result in the loss of human life.

The prime purpose of this investigation was to study the dynamics and properties of bacteria and a few selected microbicides in oil and oil-water systems under various test conditions.

CHAPTER I

INTRODUCTION

One of the first problems encountered in this investigation was the lack of suitable methods for determining the presence or absence of viable bacteria in the oil phases of oil and water combinations. The following experiments were done in an effort to evaluate some of the existing techniques or to develop improved techniques.

MEDIA AND CULTURES EMPLOYEDCultures

The microorganisms employed in these studies were; Staphylococcus aureus F.D.A. 209 (United States Food and Drug Administration), Pseudomonas fluorescens O.A.C. 99 and Pseudomonas fluorescens O.A.C. 100 (Ontario Agricultural Collection).

Media Employed

Most of the studies performed during this section of the investigation employed a medium which allowed maximal growth of the test organisms. No attempt was made to select a medium which allowed greater pigment production or resulted in the induction of particular enzyme systems.

Bacto Nutrient Broth

Peptone 5 gm

Beef extract 3 gm

Distilled water add to 1000 ml

CHAPTER I

Techniques for Determining Viable Bacteria
in Oil Phases

Trypticase Soy Broth (Baltimore Biological Laboratory)grams per litre

Trypticase (Pancreatic Digest of Casein)	17.0
Phytone (B.B.L. Soy Peptone)	3.0
Sodium chloride	5.0
Dipotassium phosphate	2.5
Dextrose	2.5

Both broths were prepared according to instructions and sterilized by autoclaving.

Solid media was obtained by the addition of 1.5 per cent Difco agar to the medium prior to sterilization.

Minimal Medium

Czapek's Modified Salts Medium (Thom and Church, 1926)

grams per litre

Saccharose	30
Sodium nitrate	3
Dipotassium phosphate	1
Magnesium sulphate	0.5
Potassium chloride	0.5
Ferrous sulphate	0.01

The above components were dissolved in 1000 ml of demineralized water. The final pH was 7.3. Sterilization was by membrane filter filtration.

When ascertaining the hydrocarbon breakdown ability for the test organisms, salts medium was employed in which the saccharose was omitted and 5 per cent light liquid petrolatum added in its place.

Preparation of Inoculum

The inoculum was prepared by growing the test organisms in broth at 37°C for 24 hours. The inoculum was washed 3 times with sterile distilled water. Each time the pellet was resuspended and centrifuged at about 10,000 r.p.m. for 5 minutes. After the third washing the cells were resuspended in sterile distilled water to an optical density of about 1, as measured in a Bausch and Lomb Spectronic 20 photometer. Surface plate counts were made from the optically measured suspensions.

Techniques Employed to Determine the Presence of Viable Bacteria in Oil

One of the greatest difficulties involved in working with hydrocarbons is the lack of adequate methods for isolating and counting bacteria from oil samples. Several techniques were tried including:

- a) reduction of tetrazolium chloride as an indication of the presence of viable organisms
- b) membrane filtration and subsequent colony development on the membrane
- c) study of the migration of the test organism from a hydrocarbon phase to an aqueous phase and subsequent growth of the microorganisms in suitable media.

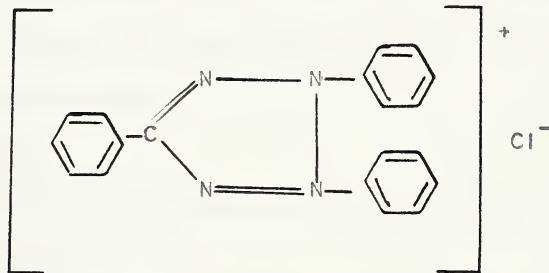
Our experience with each procedure will be discussed individually.

Tetrazolium Chloride (2,3,5 triphenyl-tetrazolium chloride)

(T.T.C.)

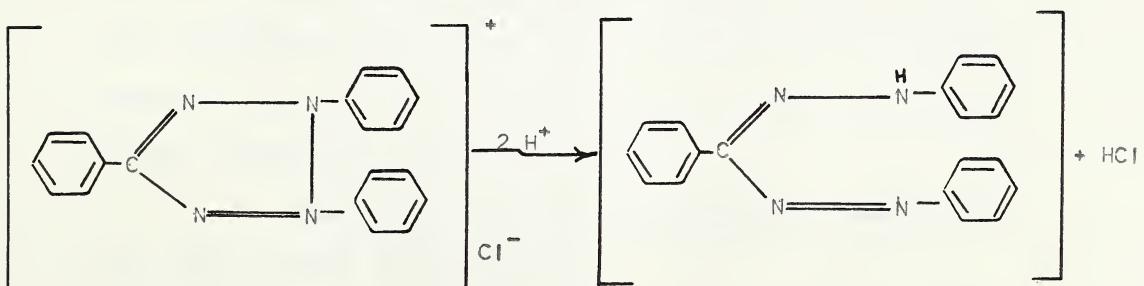
2,3,5 triphenyl-tetrazolium chloride (molecular weight - 334.81) is a pale yellow powder which darkens on exposure to light. It has a melting point of 239-241°C; readily soluble in water, methanol, ethanol and slightly soluble in acetone and chloroform, almost insoluble in diethyl ether.

Structure



2,3,5 triphenyl-tetrazolium chloride

All tetrazolium salts are characterized by their behavior upon reduction. While powerful reducing agents break down the molecule to small fragments milder reducing agents give colored formazans: (Tetrazolium Salts B.D.H.)



Triphenyl-formazan (red)

The action of ultra-violet light and to a lesser degree, of daylight, on aqueous solutions of T.T.C. produces two products, namely the formazan (which explains the development of an incipient red color) and an oxidation product.

A possible application of 2,3,5-triphenyl-tetrazolium chloride to the present problem lies in the detection of viable bacteria by the reduction of tetrazolium chloride through the activity of enzymes possessed by such cells. T.T.C. acts as a hydrogen acceptor in the presence of enzyme dehydrogenases and is reduced to the red formazan derivative.

Procedure

Two solutions of 2,3,5-triphenyl-tetrazolium chloride were prepared:

0.3% T.T.C. in chloroform

0.5% T.T.C. in deionized water

The chloroform solution of T.T.C. was prepared and a sample of light liquid petrolatum, which contained test organisms, was added forming a solution of chloroform, T.T.C. and oil. In this way the T.T.C. should have been in contact with any organisms which were suspended in the oil and reduction should have occurred if the organisms were metabolically active.

The above mixture was shaken well then incubated at 37°C and read after 24 and 36 hours.

The results of these experiments are shown in

TABLE I.

TABLE I

Determination of Viable Bacteria Using
2,3,5-Triphenyl-tetrazolium Chloride

Sample Number	Amount of oil containing bacteria (ml)	Amount of 0.3% T.T.C. in CHCl ₃ (ml)	Color development after*	
			24 hours	36 hours
1	1	3	Nil	Nil
2	1	3	"	"
3	1	3	"	"
4	1	3	"	"
5	1	3	"	"
6	1	3	"	"

* There was no color development in the test mixtures when compared to a control tube which contained 1 ml of mineral oil in place of the mineral oil containing bacteria.

The fact that no development of color occurred in any of the test systems suggests several possibilities:

- 1) the chloroform killed all the organisms and in so doing, disrupted the dehydrogenase enzyme systems necessary for conversion of T.T.C. to the corresponding formazan
- 2) the organisms in the oil are metabolically quiescent
- 3) the number of organisms in the hydrocarbon sample were too few to be detected by this method.

Only the first possibility was checked, that is, does the chloroform kill the bacteria and how quickly. TABLE II confirms the fact that chloroform kills Pseudomonas O.A.C. 99 in less than 5 minutes.

To do this we suspended bacteria in 10 ml of light liquid petrolatum, added 10 ml of 0.3% T.T.C. in chloroform and mixed quickly. Samples were removed at regular intervals and placed in trypticase soy broth. The broth was incubated, with shaking, for 24 hours.

The results of these experiments are shown in TABLE II.

TABLE II

Effect of Chloroform on Viability of
Pseudomonas O.A.C. 99 Cultures

Sample	Time of Exposure to CHCl_3 (minutes)	Growth of Organism*
Control	0	+
a	5	0
b	10	0
c	20	0
d	25	0
e	30	0
f	40	0

* Presence or absence of growth was noted by removing 0.1 ml of broth and incubating on trypticase soy agar plate.

Discussion

It is evident that T.T.C. cannot be used as an indicator of viable cells so long as it is dissolved in chloroform. We have used T.T.C. as a compensatory test of a disinfectant's ability to inhibit growth. We added 0.1 ml of a 0.5% aqueous T.T.C. solution to 5 ml of broth culture and incubated another 4 hours.

If no color change is evident and no turbidity of the culture medium, the results are taken as negative.

It must be remembered that when the tetrazolium test is used to establish the effectiveness of germicides, the principles which apply in the customary phenol coefficient test are not valid since small numbers of bacteria do not give noticeable changes in color. The sensitivity of the test lies in about 1 - 10 million organisms per ml, dependent on the luminousness. (British Drug Houses, p. 13)

Membrane Filter Technique

As has been stated in the manual of Proposed Procedures for Microbiological Examination of Fuels, " varying degrees of success have been reported with the use of this technique". When working with hydrocarbon fuels, it is essential to flush the hydrocarbon from the membrane with the aid of a nontoxic wetting agent. Hydrocarbon remaining in the membrane prevents an orderly migration of the nutrient medium through the membrane and little or no colony growth will appear on it. (Sim 1963)

Procedure

This technique involves the treatment of the oil sample with a 0.1% aqueous alkylaryl polyether alcohol* solution. The hydrocarbon may be placed in the filter apparatus and flushed through with portions of surface active agent. This procedure is repeated until all the hydrocarbon has been washed out of the filter. Next, residual surface active agent is removed with sterile distilled water.

* Marketed as Triton X-100 by the Rohm and Haas Co., Philadelphia 5, Pa.

An alternate method which is useful when working with very viscous hydrocarbons is to prepare an emulsion of the hydrocarbon in 0.1% aqueous Triton X-100. To prepare the emulsion a hand homogenizer made of stainless steel and aluminum alloy, and consists of a cup, cylinder and hand-operated pump, which forces the liquid to be homogenized through a small orifice was used.

The above procedure was tested on hydrocarbons of various composition and viscosity. Some Alberta crude oils, which had been inoculated with bacteria three years previously were also sampled by the membrane filtering procedure.

The membrane filters used, had an average pore diameter of 0.45 μ . Passage of materials through the filter was aided by the application of negative pressure.

Results

1 Cyclohexane - passes through the filter unaided by Triton X-100

2 Pentane - passes through the filter unaided by Triton X-100

3 Xylene - passes through the filter unaided by Triton X-100

4 Carbon Tetrachloride - passes through the filter unaided by Triton X-100

5 Chloroform - passes through the filter unaided by Triton X-100

6 Hexane - passes through the filter unaided by Triton X-100

7 Light Liquid Petrolatum - will not pass through the membrane filter without the aid of Triton X-100 and even in the presence of a large excess of surface active agent, only a small amount of oil will pass through before plugging occurs.

8 Esso Marvelube Motor Oil SAE 20-20W - will pass through membrane quite slowly but efficiently when emulsion is formed.

9 Mazola Corn Oil (Unsaturated) - only a very small amount will pass through even when large amounts of surface active agents are present.

10 Alberta Crude Oils

The results of these experiments are shown in

TABLE III.

TABLE III

Enumeration of Bacteria in Oil Samples Using the Membrane Filter Technique

Crude Oil Type	Test Organism per ml of Crude Oil				
	<u>Staph.</u> <u>aureus</u>	<u>209</u>	<u>B.subtilis</u>	<u>pyogenes</u>	<u>S.peorea</u>
Staph. SM11					
Joseph Lake	0		2	5	2
Wainwright	0		0	0	0
Lloydminster			Sample too viscous		
Blairmore	0		3	0	9
Peanut Oil	2		0	10	0
Normanville	0		0	++	2
Light Liquid Petrolatum	4		2	6	2
Cod Liver Oil	0		0	0	0

++ indicates too many to count

The procedure which was found most effective for the treatment of crude oils is as follows.

One ml of crude oil is added to 100 ml of 0.1% Triton X-100 and emulsified. The emulsion is then passed through the membrane filter with the aid of a vacuum pump which is attached to a special vacuum flask. Additional volumes of Triton X-100 solution are added until the membrane appears completely free of residual hydrocarbon.

When this procedure is completed, the filter is removed with sterile forceps and placed on a suitable culture medium.

Method Involving Migration of Bacteria from an Oil Phase into an Aqueous Phase

DeGray and Killian (1962) have shown that life is possible in essentially anhydrous media. These workers have shown that a hydrocarbon sample which has acquired microorganisms able to live within the hydrocarbon, is capable of a population explosion when an aqueous phase is encountered. There seems to exist a dynamic equilibrium between life in the water phase and life in the hydrocarbon phase, with constant transfer in both directions across the interface. As acetic acid will distribute itself between benzene and water, so microorganisms will distribute themselves between water and hydrocarbon. Thus the picture of two-way traffic across the interface, resulting in the establishment of a dynamic equilibrium between the populations in the two phases.

Mudd and Mudd (1924, 1926 and 1927) and Reed and Rice (1931) did some of the very early work on the behavior of non-acid-fast and acid-fast bacteria in oil and water systems.

The possible application of these observations to the present problem lies in the detection of viable bacteria in an oil sample by the formation of a two-phase, oil and water system so that the bacteria can migrate from the hydrocarbon phase into the aqueous layer and subsequently multiply there. By periodically sampling the water bottom, an estimation can be made as to whether or not bacteria are in the hydrocarbon top. This method is useful if you are not concerned with the actual number of bacteria in the hydrocarbon but rather the fact that viable bacteria are or are not present in the sample. However, in order to determine whether or not bacteria can migrate from oil to water it is necessary to devise a method of suspending the bacteria in the oil in the absence of water. Lyophilization naturally suggests itself for this purpose and the following is a description of experiments designed to investigate the possible application of lyophilization for this purpose.

The test organisms were cultured as outlined previously, washed in sterile demineralized water and resuspended in distilled water to an optical density of about 1 as measured in a Bausch and Lomb Spectronic 20 photometer at a wavelength of 600 $\text{m}\mu$. Viable counts were made of the O.D.1 suspensions enabling a check to be made on viability of the cultures after lyophilization.

Two ml of the test organism suspension were injected, with a sterile hypodermic syringe, into sterile lyophilization tubes. The suspension was quickly frozen by immersion of the tube and contents in an acetone-carbon dioxide mixture. Swirling of the tube

allowed a more rapid freezing and a greater ice-surface area in the lyophilization tube. After freezing, the tube was then connected to a vacuum line of a Vir-Tis Manifold style Freeze-Drying apparatus. The length of time required for complete drying varied with the amount of vacuum in the system and the surface area of the frozen bacterial suspension.

When the contents of the tubes were completely lyophilized, the tubes were removed from the apparatus. To determine the effect of the procedure itself on the viability of the test organism, 2 tubes of each of the test organisms were then sealed with an oxygen torch resulting in an air tight ampoule. To each of the remaining tubes 2 ml of light liquid petrolatum was added and the tubes were then sealed.

Twenty-four hours after sealing, the ampoules, which did not contain light liquid petrolatum, were opened. The organisms resuspended in 2 ml of distilled water and viable counts made on agar plates.

TABLE IV shows the effect of lyophilization on the viability of cultures of Pseudomonas fluorescens O.A.C. 99, Pseudomonas fluorescens O.A.C. 100, and Staphylococcus aureus F.D.A. 209. These results are expressed in colony forming units (cfu) rather than individual bacteria since Staphylococcus at least, tends to clump.

TABLE IV

The Effect of Lyophilization on Viability of
Pseudomonas fluorescens O.A.C. 99, O.A.C. 100,
Staphylococcus aureus F.D.A. 209

Test Organism	*cfu/ml pre-lyophilization	*cfu/ml post-lyophilization	% Survivors
<u>Pseudomonas fluorescens</u> O.A.C. 99	a) 1.67×10^9 1.60×10^9	2.0×10^8 1.53×10^8	8.4% 10.5%
	b) 1.40×10^9 1.48×10^9	1.35×10^8 1.40×10^8	10.4% 10.6%
<u>Pseudomonas fluorescens</u> O.A.C. 100	a) 1.0×10^9 1.3×10^9	2.0×10^8 2.5×10^8	20.0% 19.2%
	b) 9×10^8 1.1×10^9	1.7×10^8 1.7×10^8	18.9% 15.5%
<u>Staphylococcus aureus</u> F.D.A. 209	a) 1.30×10^9 1.28×10^9	1.27×10^8 1.27×10^8	10.2% 10.1%
	b) 1.35×10^9 1.38×10^9	1.3×10^8 1.42×10^8	10.4% 9.7%

*cfu = colony forming units

Discussion

The results show that lyophilization causes a considerable decrease in bacterial populations. This is probably due to irreversible metabolic injury to the organisms (Postgate and Hunter, 1963). Increased incubation times were used for the post-lyophilized organisms in order to enumerate those bacteria which may have suffered from "metabolic shock".

At various time intervals after sealing, ampoules containing bacteria in light liquid petrolatum have been opened and the viability of the test organisms checked using both the membrane filter technique and the migration methods previously described.

The results of these experiments are shown in

TABLE V.

TABLE V

Viability of Bacteria in Light Liquid Petrolatum
after Lyophilization

Test Organism and Date Lyophilized in Oil	Date Sampled	*Survival Based on:	
		Migration Technique	Membrane Filter Technique
<u>Staphylococcus aureus</u> F.D.A. 209 (August 1963)	August 1963	+	+
	January 1964	+	+
	March 1964	+	+
	May 1964	+	+
	July 1964	+	0
	September 1964	+	+
	November 1964	0	0
	January 1965	0	0
 <u>Pseudomonas fluorescens</u>			
O.A.C. 99 (September 1963)	September 1963	+	+
	November 1963	+	0
	January 1964	+	+
	March 1964	+	+
	May 1964	+	+
	July 1964	+	+
	September 1964	0	0
	November 1964	0	0
	January 1965	0	0

* The results denote only the presence or absence
of growth with no attempt at quantitation.

Having demonstrated that lyophilization is a suitable procedure for suspending bacteria in oil, in the absence of water, it was now possible to proceed to the investigation of migration of bacteria from hydrocarbon to water.

In order that samples of the aqueous phase of an oil-water mixture may be removed from beneath the oil phase without carrying over small amounts of hydrocarbon, it was necessary to devise a special culture apparatus which is shown in Figure I and Plate I.

Special Culture Apparatus for Oil-Water Mixtures

Figure I

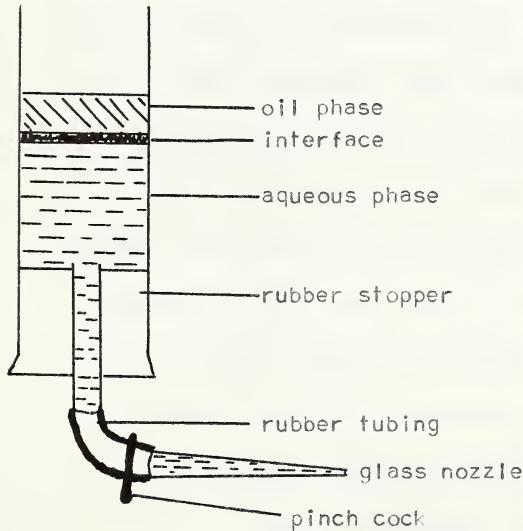
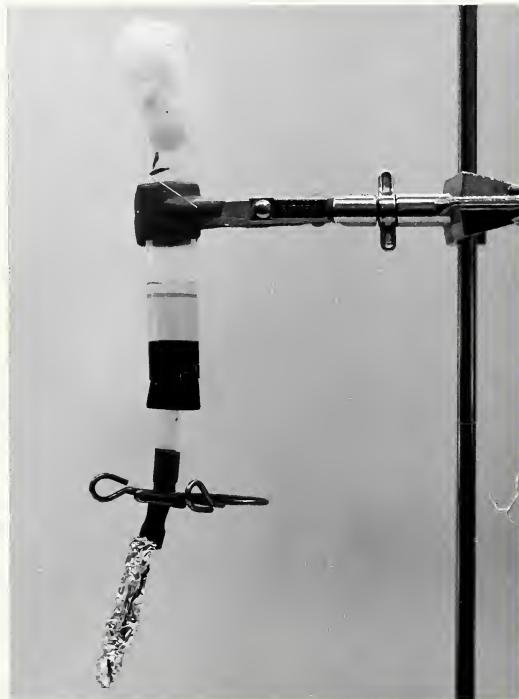


Plate I



The entire apparatus is autoclaved and the glass nozzle, through which samples of the aqueous phase are removed, is wrapped in aluminum foil so that it remains uncontaminated when not in use. Sterile mineral-salts medium (10 ml) is placed in the apparatus and 2 ml of the hydrocarbon sample containing bacteria added. At various intervals of time thereafter, the oil and water phases were mixed by replacing the cotton plug with a sterile rubber stopper and shaking the apparatus by hand. The rubber stopper is then removed and replaced with sterile cotton.

When a viable count is to be made on the aqueous phase, the total aqueous layer is removed and collected in a sterile Erlenmeyer (50 ml) flask. Removal of the entire aqueous layer avoids possible loss of microorganisms which may settle into the glass nozzle or accumulate at the oil-water interface, where growth is most profuse. Once removed, the total aqueous phase is shaken vigorously to obtain an even distribution of the organisms in the water and plate counts are done using trypticase soy agar medium. The volume of aqueous phase remaining after removal of the sample for the plate count is aseptically returned to the special culture apparatus and left in contact with the hydrocarbon until another count is to be made.

Migration Method as Used with Special Sampling Apparatus

Using lyophilized samples of bacteria in light liquid petrolatum containing the following number of organisms per 2 ml respectively; Staphylococcus aureus F.D.A. 209 1.6×10^7 /2 ml, Pseudomonas fluorescens O.A.C. 99 1.6×10^7 /2 ml, and Pseudomonas fluorescens O.A.C. 100 1.2×10^7 /2 ml, the contents of a single

ampoule (2 ml) was added to 10 ml of mineral-salts medium (Czapek's) in the special apparatus and shaken as mentioned previously. Mixtures were incubated at room temperature (20-25°C). Enumeration of viable cells in the aqueous phase of each mixture was done at 7 days and 10 days incubation by surface inoculation of 0.1 ml to trypticase soy agar for Pseudomonas and sheep blood agar for Staphylococcus.

The data from these experiments are shown in TABLE VI.

TABLE VI

Migration of Bacteria from Hydrocarbon to
Aqueous Phase of an Oil-Water Mixture

Test Organism and Oil-Water Contact Time	Sample Number	Bacterial Content of:*	
		Hydrocarbon Phase	Aqueous Phase
<u>Staphylococcus aureus</u> F.D.A. 209	1	8×10^6	0
Prior to mixing		-	6.4×10^7
7 days after mixing		-	6.4×10^7
10 days after mixing		-	
<u>Staphylococcus aureus</u> F.D.A. 209	2	8×10^6	0
Prior to mixing		-	4.0×10^7
7 days after mixing		-	4.4×10^7
10 days after mixing		-	
<u>Pseudomonas fluorescens</u> O.A.C. 99	1	8×10^6	0
Prior to mixing		-	2.5×10^7
7 days after mixing		-	2.8×10^7
10 days after mixing		-	
<u>Pseudomonas fluorescens</u> O.A.C. 99	2	8×10^6	0
Prior to mixing		-	3.6×10^7
7 days after mixing		-	4.0×10^7
10 days after mixing		-	
<u>Pseudomonas fluorescens</u> O.A.C. 100	1	6×10^6	0
Prior to mixing		-	2.8×10^7
7 days after mixing		-	3.2×10^7
10 days after mixing		-	

TABLE VI Cont.

Test Organism and Oil-Water Contact Time	Sample Number	Bacterial Content of:*	
		Hydrocarbon Phase	Aqueous Phase
<u>Pseudomonas fluorescens</u>			
O.A.C. 100	2		
Prior to mixing		6×10^6	0
7 days after mixing		-	1.0×10^8
10 days after mixing		-	8.4×10^7

* Results expressed in colony forming units per ml

and values are an average of three readings.

- Indicates no readings taken.

In each experiment, a control was included which consisted of light liquid petrolatum and mineral salts medium. No growth developed in any of the controls during the time of the experiment.

These results indicate that the bacteria survived in the essentially non-aqueous environment (lyophilized and suspended in oil) until favorable conditions for growth were encountered (contact with an aqueous medium).

When the hydrocarbon containing the bacteria came in contact with a mineral salts medium, the bacteria migrated from the oil into the aqueous phase with subsequent multiplication in the aqueous phase. Since the only carbon source available to the organisms was the components of light liquid petrolatum, the bacteria must have utilized some component or components of the oil.

It is known that most organisms migrate and grow in the area of the oil and water interface (Prince, 1961). To determine if any organisms remained dispersed throughout the oil, in the following experiments 0.5 ml of hydrocarbon phase was removed from the special culture apparatus, which had been incubated for 10 days,

and added to sterile mineral salts medium of a second culture apparatus.

Plate counts were made on the aqueous phase of the mixture after 10 days incubation at room temperature (about 22-25°C). The aqueous phases of Staphylococcus aureus, Pseudomonas O.A.C. 99 and Pseudomonas O.A.C. 100 contained respectively 1.0×10^7 cfu/ml, 5×10^6 and 3.7×10^7 cfu/ml. These results are an average figure for 3 determinations.

Discussion

Although microorganisms migrated from the hydrocarbon phase into the aqueous phase during the initial 10 day period of contact, some organisms remained in the hydrocarbon phase or they may have moved from the aqueous phase back into the hydrocarbon phase by natural motility, brownian movement or convections. Regardless of the latter possibility, viable microorganisms were found in the hydrocarbon phase after the initial migration. The method by which they got into the hydrocarbon phase is not of particular consequence here but rather that they are present in the hydrocarbon phase.

CHAPTER II

A Study of Antiseptics and Disinfectants in
Oil and Oil-Water Systems

HISTORY

The study of the activity of antiseptics and disinfectants in oil and oil and water systems is not complicated in the case of anhydrous or essentially anhydrous oils since bacteria, if they grow at all, can be readily controlled by the use of oil soluble disinfectants. The problem however becomes many times more complicated when an aqueous phase is introduced to a hydrocarbon phase and either of the two phases contain microorganisms. If the hydrocarbon phase contains organisms which are quiescent, there will more than likely be a population explosion when the aqueous phase is encountered. This population explosion in the water bottom will result in larger numbers of organisms in the hydrocarbon phase as well since a constant movement appears to take place across the oil-water interface. This movement may be attributed to natural convections, motility of the organisms or agitation.

A very important consideration in the choosing of a bactericide is the relative solubility of the bactericide in water and in oil. Certain types of compounds such as the amines are more soluble in oil than in water, especially at low concentrations. When these compounds are used in systems that contain oil, poor bacterial control can be expected since the bactericide will be extracted from the water by the oil. A prime example of this was shown by Allred in which laboratory testing indicated that sodium tetra chlorophenate had a very low oil solubility and would inhibit the growth of sulfate reducers at concentrations between 5 - 10 ppm. Testing by Allred has shown that about 20 - 25% of the phenate

goes into the oil phase of the system. This means that when 15 ppm is injected into the system, only 10 - 15 ppm will be left in the water.

It is the relative solubility problem which we became interested in and most of our work involves the distribution or partitioning effects of certain inhibitors and a study of factors which influence the speed with which the partitioning occurs and factors which give a quantitative picture as to the amount of disinfectant which migrates either from oil into water as in the case of inhibitors which are both oil and water soluble or the amount which moves from water into oil as is the case with disinfectants which are more lyophilic than hydrophilic.

INTRODUCTION

For the reasons previously given, it is desirable to determine the antibacterial activity of oil soluble antiseptics, e.g. phenol, 6-chloro-thymol, amylphenol, 2,4,6-trichlorophenol and para-chloro-meta-cresol and water soluble antiseptics, e.g. phenol, benzalkonium chloride, para-chloro-meta-cresol, and poly-vinyl-pyrrolidone-iodine in oil, in water and in oil-water mixtures. Also it is of interest to study the effect of dissolving the antiseptic in one phase only and suspending the test organisms (Staphylococcus aureus F.D.A. 209, Pseudomonas fluorescens O.A.C. 99, Pseudomonas fluorescens O.A.C. 100) in the opposite phase or in same phase or in both phases.

Disinfectants and Antiseptics Employed

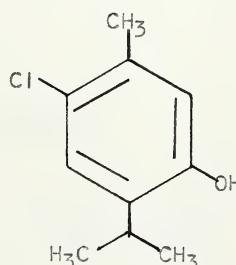
Several members of the phenolic group of disinfectants were chosen as test chemicals in our disinfection studies as well as several non-phenolic disinfectants.

The phenol derivatives were selected as a test group for several reasons:

1) The compounds used all have a basically similar structure

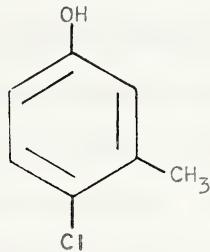
a) 6-chlorothymol

(6-chloro-4-isopropyl-1-methyl-3-phenol)

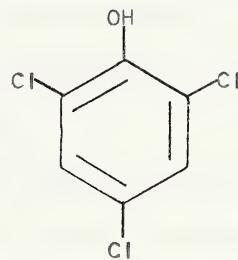


b) para-chloro-meta-cresol

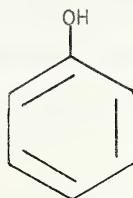
(4-chloro-3-methyl-phenol)



c) 2,4,6-trichlorophenol

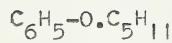


d) Phenol



e) Amyl Phenol

(p-tert-amyl phenol)



2) These compounds possess varying degrees of solubility in oil and in water phases.

- a) 6-chlorothymol-slightly soluble in water
- b) 4-chloro-3-methyl-phenol-slightly soluble in water
- c) 2,4,6-trichlorophenol-slightly soluble in water
- d) Phenol-soluble 1 in 15 parts of water
- e) Amyl Phenol-virtually insoluble in water

Phenol derivatives become less water soluble, more lipid soluble, and more bactericidal with increasing halogenation and/or alkylation. (Sykes 1958 and Reddish 1957)

3) The mechanism of action on bacterial cells are similar.

It has long been recognized that phenolic compounds act as protein precipitants and denaturants.

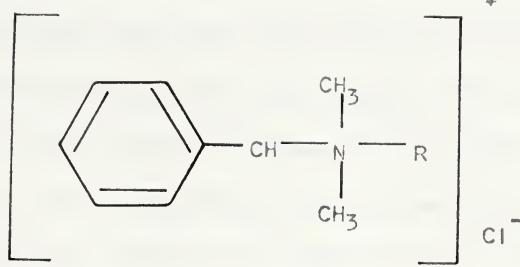
Judis (1962, 1963, 1964) has done much work concerning the release of radioactivity from C¹⁴ labelled E. coli, indicating that the phenolics exert their lethal activity by damaging the cell membrane or mechanisms which control permeability of the cell membrane. According to Judis, these compounds might inhibit respiratory enzymes thus preventing energy yielding reactions.

4) The disinfectants are commonly used in certain pharmaceutical and other industrial systems.

Along with the above mentioned phenolic disinfectants the following were also studied:

a) Benzalkonium Chloride

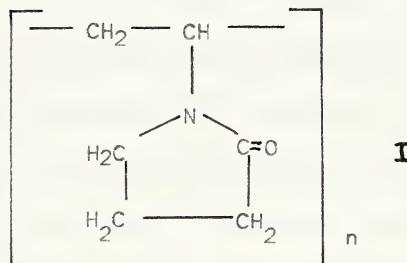
A cationic surface active agent. It is a mixture of alkyl dimethylbenzyl ammonium chlorides of the general formula in which "R" represents a mixture of the alkyls from C₈H₁₇ to C₁₈H₃₇.



The compound is very soluble in water and has a phenol coefficient at 37°C of 407 for Staphylococcus aureus.

6) Polyvinylpyrrolidone-Iodine Complex (Betadine B.D.H.)
(P.V.P-I)

A complex of polyvinylpyrrolidone and iodine which is water soluble and soluble in oil.



Methods

Studies in Aqueous Systems

Initially the antibacterial activity of a number of antiseptics and disinfectants was studied in aqueous solution to determine the approximate concentration of disinfectant necessary to kill the test organisms. Those disinfectants tested were phenol, chlorothymol, para-chloro-meta-cresol, benzalkonium chloride, polyvinylpyrrolidone-iodine and 2,4,6-trichlorophenol.

Bactericidal values were determined by pipetting 0.1 ml of a 24 hour broth culture of the test organism into sterile tubes containing 5 ml of the various dilutions of the disinfectant, then shaking well. At various time intervals, a 4 mm loopful of the mixture was removed and transferred aseptically to a tube containing 5.0 ml of sterile nutrient broth or trypticase soy broth (T.C.S. broth).

To reduce or eliminate the bacteriostatic effect of benzalkonium chloride, letheen broth was used in place of the nutrient or trypticase soy broth.

Quaternary Ammonia Neutralizing Medium (Kohn 1963)

Lecithin	0.5 gm
Tween 80	3.0 gm
Nutrient Broth	0.8 gm
Purified water q.s. ad. 100 ml	

For tests involving polyvinylpyrrolidone-iodine, sodium thiosulfate was incorporated into the broth. Broth tubes were then incubated for 48 hours aerobically at 37°C and growth or no growth recorded, as evidenced by turbidity and checked using triphenyltetrazolium chloride. Tubes showing growth were checked by Gram's stain to confirm the characteristic organism. All determinations were made in duplicate. The following results indicate the effectiveness of various disinfectants in aqueous solutions.

ResultsTest Using *Staphylococcus aureus* F.D.A. 209

- a) Phenol - a 1/80 solution of phenol in water kills in less than 5 minutes.
- b) Para-chloro-methyl-phenol - a 1/2000 solution kills in 15 minutes but not in 10 minutes.
- c) Benzalkonium chloride - a 1/50,000 solution kills in 15 minutes but not in 10 minutes.
- d) 6-chlorothymol - a 1/8000 solution kills the test organism in 15 minutes but not in 10 minutes.
- e) Betadine B.D.H. (1% available iodine) - 25 ppm of available iodine kills in 20 minutes but not in 15 minutes.

The disinfectant 6-chlorothymol was also tested on Pseudomonas fluorescens O.A.C. 99. It was found to be of no use for this organism when in aqueous solution since a high enough concentration could not be achieved to kill even after 4 hours of exposure to the disinfectant. The solubility of 6-chlorothymol in water was found to be about 1 in 6000.

For data on these findings refer to Appendix I.

Studies Involving Oil and Water Systems

Before disinfection studies could be done on two phase systems, it was necessary to find a test system whose parameters could be used as bases for subsequent work. Bennett (1962) has demonstrated, using Pseudomonas aeruginosa cells, that the oil-water ratio has a significant effect upon the magnitude of microbial

growth in metal-cutting emulsions. In one particular experiment Bennett has shown that a 1:5 oil-water ratio is inhibitory and normally there is no growth or very little growth, and a 1:10 ratio is partially inhibitory which results in a prolongation of the lag phase; however, the organisms gradually adapt themselves to the environment and after several days they produce relatively good growth. The 1:25 and 1:50 ratios are almost invariably the most ideal for maximum growth. In ratios greater than 1:50, the inhibitory components are diluted to a point where they are no longer active and the concentration of oxidizable hydrocarbons seems to be the major limiting factor.

Our studies in this regard follow.

Effect of the Oil-Water Ratio on Microbial Growth

Organisms in the Oil Phase

To demonstrate the effect equal quantities of test organism in mineral oil (by lyophilization procedure) were inoculated into a series of flasks containing different quantities of sterile water. This mixture was placed in 125 ml Erlenmeyer flasks and incubated at 37°C while shaking on a mechanical wrist-action shaker. A control group was carried at 37°C but without shaking.

The results from this and other experiments which involved attempts at quantitative recovery of organisms from oil were not successful. We were unable to show any correlation between the oil-water ratio and microbial growth and it became even more obvious that a quantitative recovery of organisms, using freeze-dried organisms suspended in oils, is impossible using this procedure. This may be due to the clumping effect of the organisms and isolation of the clumps from the nutrient aqueous phase by a

thin-layer of oil. According to Raymond and Davis (1960) in their investigation of n-alkane utilization and lipid formation by a soil isolate of the genus Nocardia, the cells become imbedded in the oil phase out of sufficient contact with the required aqueous phase to allow growth. Their method of counting also yielded low counts which was probably due to large masses of cells, because of their occlusion and adsorption by the liquid alkane globules, being the foci of individual colonies.

Organisms in the Aqueous Phase

Since insignificant results were obtained when the organisms were suspended in the oil phase, the test was changed so that the test organisms, Staphylococcus aureus F.D.A. 209 and Pseudomonas fluorescens O.A.C. 99, were in the aqueous phase and the oil content was varied.

Twenty-four hour trypticase soy broth cultures of the test organisms were harvested by centrifugation at 10,000 r.p.m. for 5 - 10 minutes, washed with sterile distilled water twice then counted by the serial dilution and plate count method. Trypticase soy agar plates were incubated at 37°C for 24 hours.

Procedure and Methods

The following ratios were prepared, 5%, 10%, 25% and 50% hydrocarbon that is, of the total 50 ml of oil and water, 5%, 10%, 25% and 50% of this total is light liquid petrolatum. This system was prepared for two test organisms and for shaking and stationary cultures.

The aqueous phases contained at time zero:

- a) Staphylococcus aureus - 1.34×10^8 organisms/ml
- b) Pseudomonas fluorescens - 3.7×10^8 organisms/ml

A control was carried which contained no hydrocarbon. At intervals of 3 days, samples of the aqueous phase were removed from the culture vessels. Plate counts were made on these samples.

TABLE VII, VIII, IX, X and GRAPHS I, II, III, and IV show the results of these experiments.

TABLE VII

Effect of Oil to Water Ratio on Growth of Pseudomonas

fluorescens O.A.C. 99 in Shaking Culture*

Time (days)	% Light Liquid Petrolatum				
	5%	10%	25%	50%	Control
0	3.7×10^8	3.7×10^8	3.7×10^8	3.7×10^8	3.7×10^8
3	1.42×10^9	1.46×10^9	1.64×10^9	1.11×10^9	2.8×10^8
6	4.0×10^7	4.0×10^7	1.5×10^8	3.0×10^7	1.5×10^8
9	3.8×10^7	5.0×10^7	1.13×10^8	1.14×10^8	3.0×10^7

* Results expressed in colony forming units per ml.

TABLE VIII

Effect of Oil to Water Ratio on Growth of Pseudomonas

fluorescens O.A.C. 99 in Stationary Culture*

Time (days)	% Light Liquid Petrolatum				
	5%	10%	25%	50%	Control
0	3.7×10^8	3.7×10^8	3.7×10^8	3.7×10^8	3.7×10^8
3	1.38×10^9	1.56×10^9	1.52×10^9	1.52×10^9	3.0×10^8
6	3.5×10^8	4.2×10^8	6.0×10^7	1.1×10^8	2.0×10^8
9	1.36×10^8	1.7×10^8	9.5×10^7	7.5×10^7	1.2×10^8

* Results expressed in colony forming units per ml.

TABLE IX

Effect of Oil to Water Ratio on Growth of
Staphylococcus aureus F.D.A. 209
 in Stationary Culture*

Time (days)	% Light Liquid Petrolatum				
	<u>5%</u>	<u>10%</u>	<u>25%</u>	<u>50%</u>	<u>Control</u>
0	1.34×10^8	1.34×10^8	1.34×10^8	1.34×10^8	1.34×10^8
3	3.5×10^6	4.5×10^6	3.0×10^6	4.0×10^6	4.0×10^6
6	4.0×10^6	4.0×10^6	2.0×10^6	1.0×10^5	3.0×10^6
9	5.6×10^6	7.0×10^6	5.1×10^6	1.1×10^6	3.0×10^6

* Results expressed in colony forming units per ml.

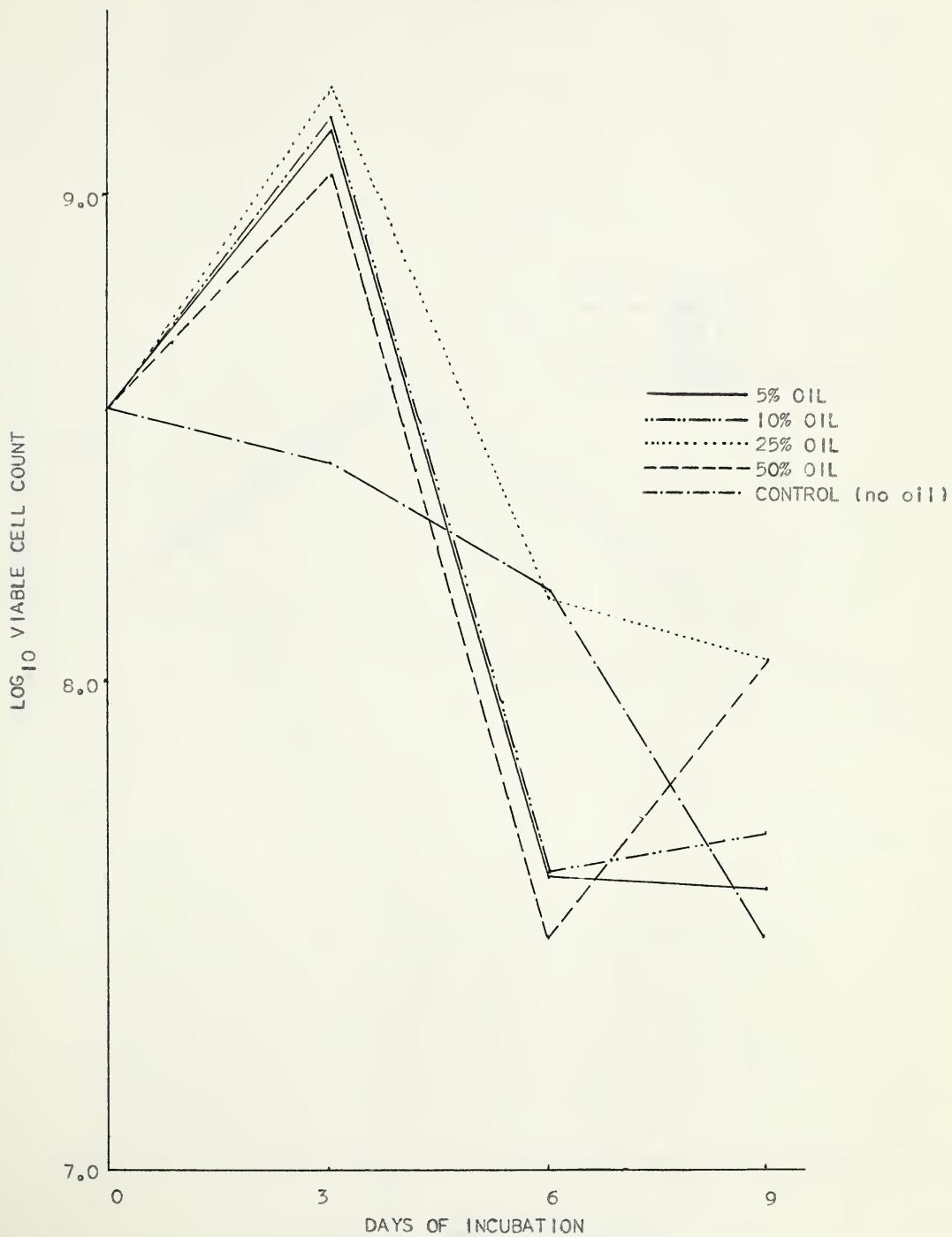
TABLE X

Effect of Oil to Water Ratio on Growth of
Staphylococcus aureus F.D.A. 209
 in Shaking Culture*

Time (days)	% Light Liquid Petrolatum				
	<u>5%</u>	<u>10%</u>	<u>25%</u>	<u>50%</u>	<u>Control</u>
0	1.34×10^8	1.34×10^8	1.34×10^8	1.34×10^8	1.34×10^8
3	2.0×10^6	3.5×10^6	3.0×10^6	2.5×10^6	3.5×10^6
6	4.0×10^5	1.6×10^6	2.0×10^6	2.0×10^5	1.0×10^6
9	1.1×10^6	1.8×10^6	1.0×10^5	1.0×10^3	1.0×10^5

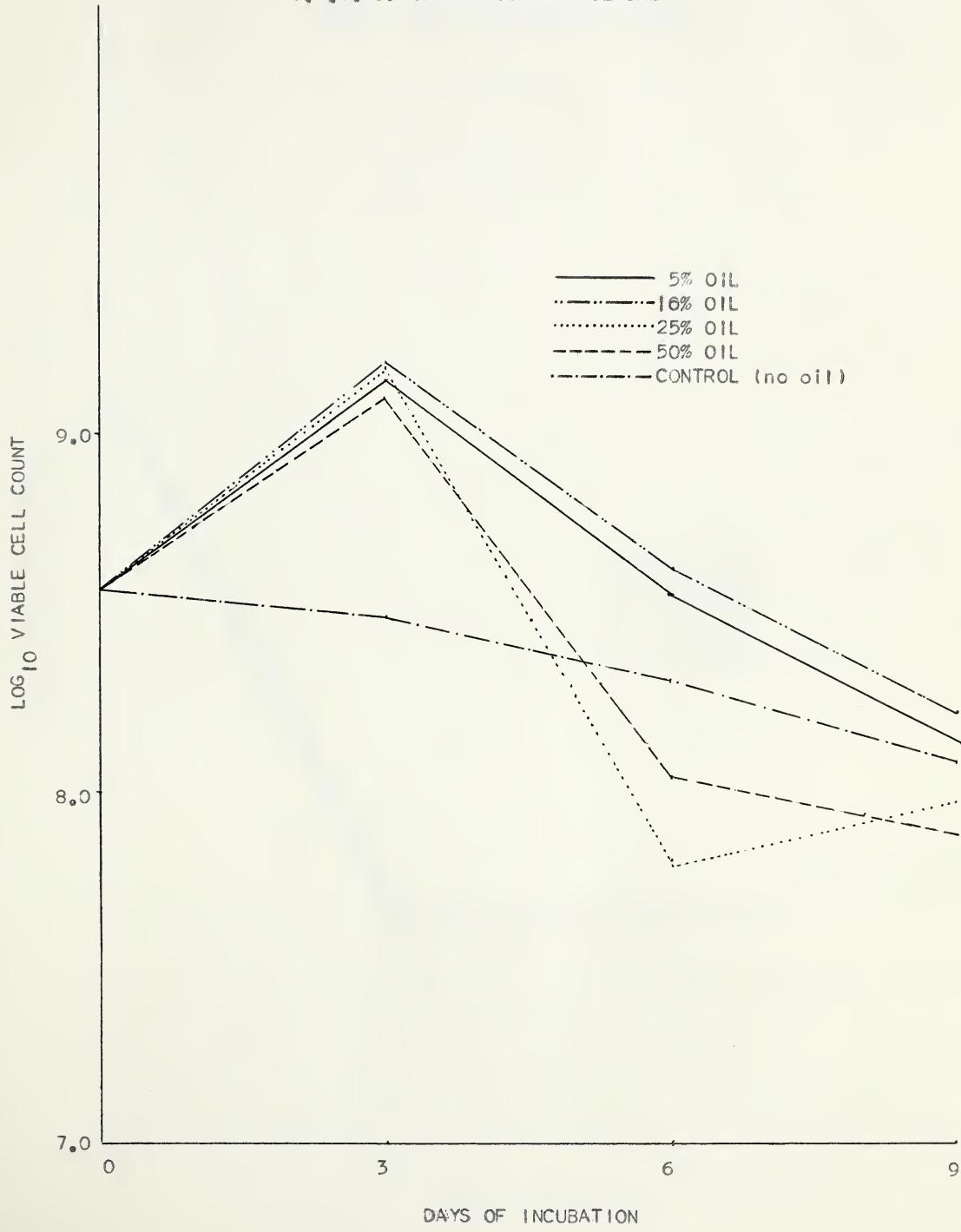
* Results expressed in colony forming units per ml.

The following graphs will serve to demonstrate these results more effectively.

GRAPH IEFFECT OF OIL TO WATER RATIO ON GROWTH OF PSEUDOMONAS FLUORESCENSO_A, C₉ IN SHAKING CULTURE

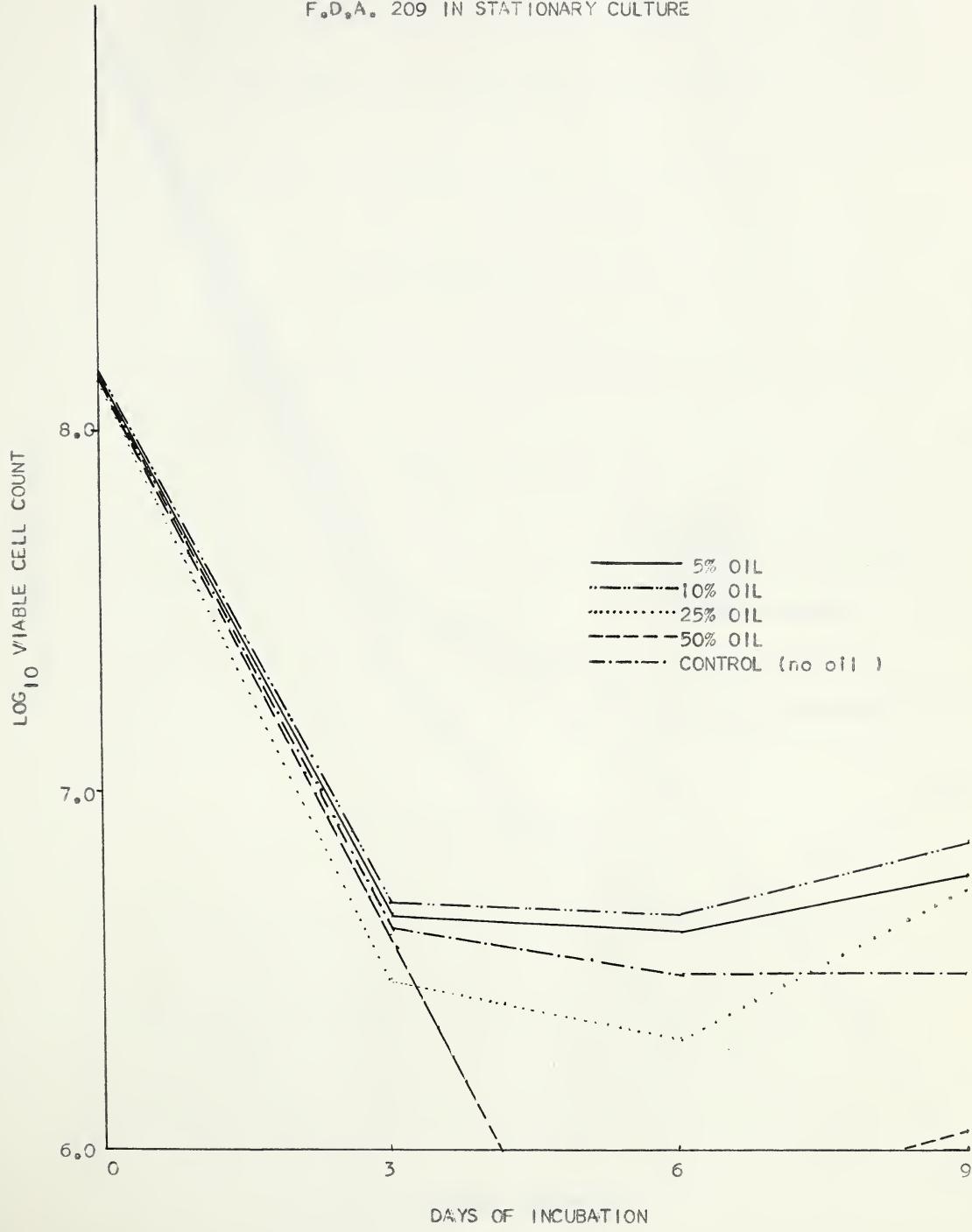
GRAPH II

EFFECT OF OIL TO WATER RATIO ON GROWTH OF PSEUDOMONAS FLUORESCENS
O₉A₉C₉ 99 IN STATIONARY CULTURE



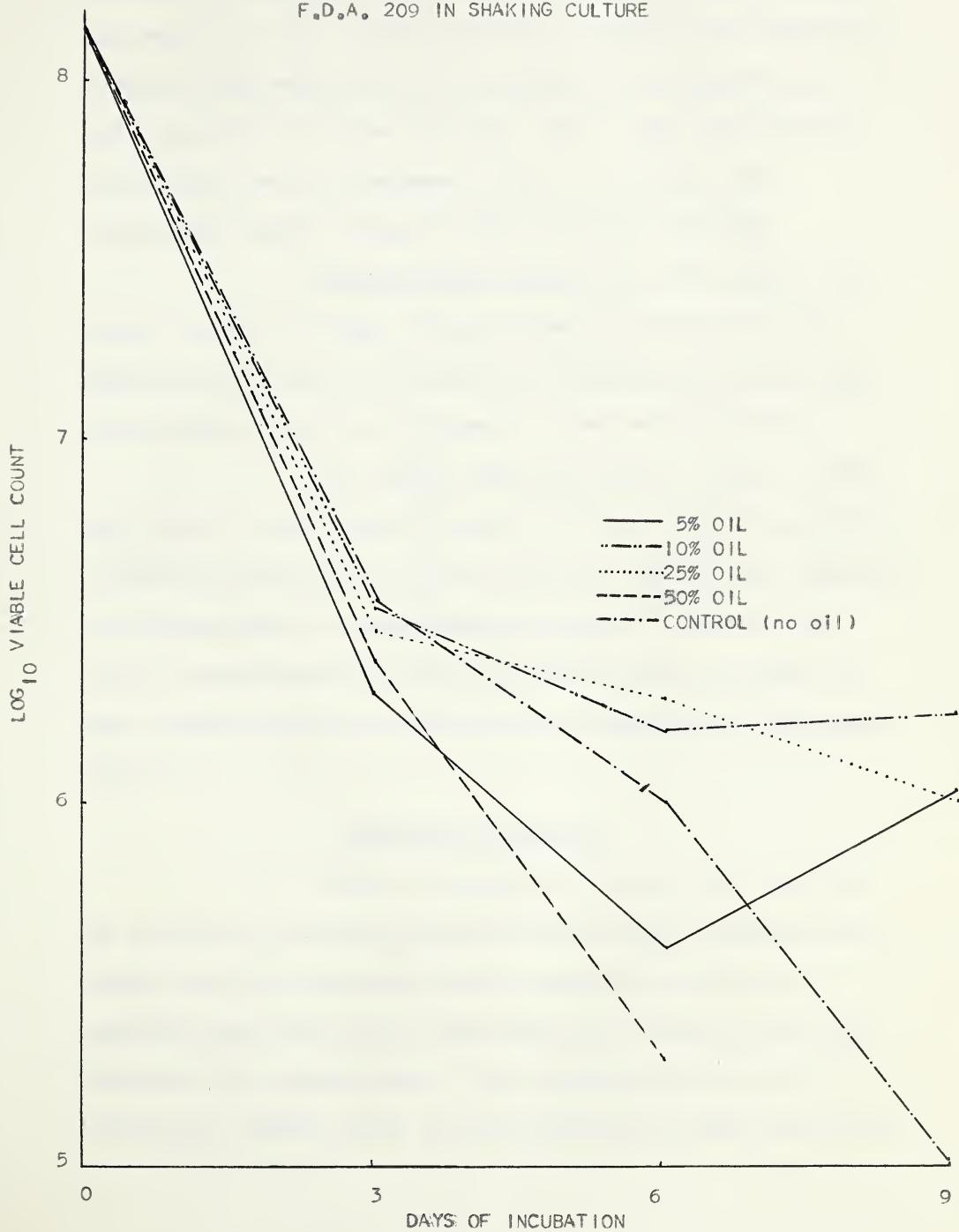
GRAPH III

EFFECT OF OIL TO WATER RATIO ON GROWTH OF STAPHYLOCOCCUS AUREUS
F.D.A. 209 IN STATIONARY CULTURE



GRAPH IVEFFECT OF OIL TO WATER RATIO ON GROWTH OF STAPHYLOCOCCUS AUREUS

F.D.A. 209 IN SHAKING CULTURE



Discussion of Results

The preceding graphs indicate that after 3 days of incubation at 37°C, the Pseudomonas fluorescens O.A.C. 99 increased in concentration in the aqueous phase. This effect may have been due to the organisms ability to utilize some components of light liquid petrolatum in the presence of trace amounts of nutrient carry over in the inoculum. After 3 days the bacterial concentration began to decrease. Since no nutrients were intentionally added, a drop in viable count was expected.

Staphylococcus aureus F.D.A. 209 viable count dropped sharply in 3 days. This decrease was probably due to a combination of inability to utilize the hydrocarbon substrate and lysis of the cells in an environment of low osmotic pressure.

Our results agree with those of Bennett (1962) that the oil to water ratio does have an effect upon the magnitude of microbial growth in an oil-water system. After 3 days incubation the maximum growth of Staphylococcus aureus was evidenced in a 10% oil to water system. A 25% oil to water mixture provided the most suitable system for maximum growth of Pseudomonas fluorescens O.A.C. 99.

Migration of Bacteria

When 1 ml aliquots of the oil phase from both the stationary and shaking cultures (after 9 days incubation) were removed from the incubation flasks, inoculated into 20 ml of trypticase soy broth (T.C.S.) and shaken for 36 hours, growth was observed in the aqueous phase. This procedure indicates that bacteria are capable, under certain conditions, of migrating from an

aqueous to a hydrocarbon phase and then from the hydrocarbon into an aqueous phase.

In this experiment the bacteria first migrated from water into light liquid petrolatum, then from light liquid petrolatum to T.C.S. broth. This final migration was noted visually by growth in the T.C.S. broth. TABLE XI gives evidence for these observations.

TABLE XI

Migration of Bacteria from Light Liquid Petrolatum

to T.C.S. Broth as Evidenced by Visual Observation

Test Organism	% Hydrocarbon in Medium	Stationary Culture	Shaking Culture	Control*
<u>Staphylococcus aureus</u>	5%	+	+	0
" " "	10%	+	+	0
" " "	25%	0	0	0
" " "	50%	+	+	0
<u>Pseudomonas O.A.C. 99</u>	5%	+	+	0
" " "	10%	+	+	0
" " "	25%	+	+	0
" " "	50%	+	+	0

* Control was light liquid petrolatum in T.C.S. broth.

Lack of growth indicated that there was no contamination of samples while on the shaking device and that the light liquid petrolatum used for this test was not itself contaminated.

The aqueous phase from each of these tubes was plated on T.C.S. agar plates to macroscopically prove that the growth was due to the test organisms.

Mudd and Mudd (1924, 1926, 1927) made the observation that non-acid-fast bacteria in the aqueous phase of a water-oil emulsion, on reaching the interface either remain in the interface or return to the water but never pass into the oil. Acid-fast bacteria on the other hand which enter the interface from the water are snapped abruptly into the oil phase. These phase-boundary phenomena were observed under a dark field microscope.

Reed and Rice (1931) also studied the distribution of acid-fast and non-acid-fast bacteria in water-oil systems. They suspended bacteria in aqueous solution in test tubes and layered various amounts of olive oil on top of the aqueous phase. The tubes were stoppered and shaken mechanically for one hour. The emulsions were allowed to separate and concentrations of bacteria remaining in the aqueous phase was determined by comparing the opacity with that of a standard suspension of the same organism or by counting the bacteria. Their results were in agreement with Mudd's conclusions.

These earlier workers (Reed and Rice 1931, Mudd and Mudd 1924, 1926) working with both acid-fast and non-acid-fast organisms have observed that in oil and water mixtures, the acid-fast organisms tend to go into the oil phase of an oil-water mixture whereas non-acid-fast organisms tend to remain in the aqueous phase with no migration into the opposite phase.

Our studies regarding the migration of 2 non-acid-fast organisms in oil-water mixtures, namely, Staphylococcus aureus and Pseudomonas fluorescens, do not agree with the results of Mudd and Reed. We were able to recover Pseudomonas from the

hydrocarbon phases of all the systems tested and Staphylococcus was recovered from all systems except the 25% oil to water mixture. (See TABLE XI) We do not feel that the assay technique of these investigators was sensitive enough to detect the few organisms which may be in the hydrocarbon phase. The technique we employ will allow the presence of a very few bacteria (less than could probably be detected by optical density difference) to be noted due to their rapid multiplication when a suitable growth environment is encountered.

Disinfection of Hydrocarbon Phases

Preparation of Test Mixtures

As a result of our studies on the effect of oil to water ratio and the effect of incubation time on microbial growth it was decided to use, in subsequent experiments, an incubation time of 3 days and an oil concentration of 10% (oil to water) for Staphylococcus aureus F.D.A. 209 and 25% oil for Pseudomonas fluorescens O.A.C. 99. The total volume of the two phases being 50 ml's.

Preparation of the Hydrocarbon - Disinfectant Phase

The test disinfectants were dissolved in the light liquid petrolatum in concentrations which yielded 1%, 0.5% and 0.25% final concentrations of disinfectant relative to the total volume (50 ml's) of the experimental system. Thus, the initial concentration of disinfectant in the hydrocarbon was actually greater than above mentioned values.

e.g. a) For the Staphylococcus aureus test series, which contained 10% hydrocarbon, to make the disinfectant concentration 1% relative to the

total volume required 0.5 gm of disinfectant per 5 ml oil.

b) For the Pseudomonas fluorescens test series, 0.5 gm of disinfectant was added to 12.5 ml of oil.

The following disinfectants were used:

- 1) phenol
- 2) amyłphenol
- 3) 2,4,6-trichloro phenol
- 4) 6-chlorothymol
- 5) p-chloro-meta-cresol

Preparation of the Water-Bottom

The 2 test organisms were grown in trypticase soy broth at 37°C for 24 hours, then washed twice with sterile water and finally suspended in sterile water to an optical density of 0.5 as measured in a Spectronic 20 photometer at 600 m μ .

Hydrocarbon-Water Mixture

An appropriate amount of aqueous test organisms suspension (45 ml or 37.5 ml) and hydrocarbon (5 ml or 12.5 ml) were mixed and incubated at 37°C on a mechanical-wrist shaker for 3 days. The stopper used on the incubation flasks was rubber covered with aluminum foil to prevent any effects which may be due to contact between the phases and/or organism and rubber.

After 3 days the flasks were removed from the shaker and the two phases allowed to separate. In cases where separation was slow, a portion of the oil-water mixture was placed in a test-tube which increased the depth of the oil layer and thus facilitated

removal of the upper oil phase.

One ml of the oil was placed in T.C.S. broth and shaken for 36-48 hours to note if any organisms had migrated into the oil phase and survived there. At the same time, the aqueous phase was assayed for viable bacteria. If the bacteria were killed in the aqueous bottom it was due to a partitioning effect of the disinfectant from the hydrocarbon phase to the aqueous phase and/or an actual contact between the cells and the disinfectant in hydrocarbon, due to shaking.

In all cases a control was maintained which contained microorganisms in the aqueous phase and hydrocarbon without disinfectant present.

The results of these experiments are shown in TABLES XII and XIII.

TABLE XII

The Effect of Various Concentrations of Disinfectants on Staphylococcus aureus F.D.A. 209 When Disinfectant is in Hydrocarbon Phase

Disinfectant	Phase : Concen- tration:	Oil 1%	Aqueous 1%	Oil 0.5%	Aqueous 0.5%	Oil 0.25%	Aqueous 0.25%
Phenol		0	0	0	0	+	+
2,4,6-tri- chlorophenol		0	0	0	0	0	+
Amyl phenol		0	+	+	+	+	+
p-chloro- meta-cresol		0	*	*	*	0	0
6-chloro- thymol		0	0	0	+	+	+
Control		+	+	+	+	+	+

* Indicates that the disinfectant is not soluble at this concentration.

TABLE XIII

The Effect of Various Concentrations
of Disinfectants on Pseudomonas fluorescens O.A.C. 99
When Disinfectant is in Hydrocarbon Phase

Disinfectant	Phase: Concentration:	Oil	Aqueous	Oil	Aqueous	Oil	Aqueous
		1%	1%	0.5%	0.5%	0.25%	0.25%
Phenol		0	0	0	+	+	+
2,4,6-trichlorophenol		0	0	+	+	+	+
Amyl Phenol		+	+	+	+	+	+
p-chloro-meta-cresol		*	*	*	*	0	0
6-chloro-thymol		0	+	0	+	0	+
Control		+	+	+	+	+	+

* Indicates that the disinfectant is not soluble at this concentration.

Discussion of Results

Staphylococcus aureus F.D.A. 209 as Test Organism

a) Phenol - a sufficient amount of phenol migrates from the hydrocarbon phase into the aqueous phase to kill the test organism when the disinfectant concentration is 1% and 0.5% but there is no disinfectant action in either the oil or aqueous phase when the concentration is 0.25%.

b) 2,4,6-trichlorophenol - the partitioning effect is sufficient at concentrations of 1% and 0.5% to kill the test organism in both phases, but when the concentration is 0.25%, killing only occurs in the hydrocarbon phase.

c) Amyl phenol - There is no partitioning effect between phases with amy1 phenol. The organisms can survive in the aqueous phase at all concentrations tested. Staphylococcus is inhibited in the oil phase at 1% concentration but not at 0.5% or 0.25%.

d) para-chloro-meta-cresol - Only a concentration of 0.25% was tested since this concentration was the limit of solubility for this disinfectant. It was found effective in both phases.

e) 6-chlorothymol - Partitioning was sufficient at 1% to inhibit the test organism in both phases. At 0.5% there was inhibition only in the oil phase. At 0.25%, growth occurred in both phases.

Pseudomonas fluorescens O.A.C. 99 as Test Organism

a) Phenol - The partitioning effect is sufficient with a concentration of 1% phenol to kill in both phases but at 0.5% there is inhibition only in the oil phase. No inhibition occurs in either phase at a concentration of 0.25%.

b) 2,4,6-trichlorophenol - At a concentration of 1%, inhibition occurs in both phases but at 0.5% and 0.25%, there is inhibition in neither phase.

c) Amyl phenol - Does not inhibit Pseudomonas in either phase at any concentration employed.

d) para-chloro-meta-cresol - As with the Staphylococcus test system, only one concentration was tested due to the limited solubility of this inhibitor. At 0.25% the partitioning effect was sufficient to kill the test organism in both phases.

e) 6-chlorothymol - This disinfectant is of no use for inhibiting O.A.C. 99 in the aqueous phase even at 1% concentrations but is effective at 1%, 0.5% and 0.25% in the hydrocarbon phase.

From the foregoing results it is evident that two forms of migration occurred during these experiments.

- 1) The disinfectant migrated from the hydrocarbon phase to the aqueous phase.
- 2) The bacteria migrated from the aqueous phase into the hydrocarbon phase. With these 2 test organisms, this migration would probably not occur naturally but was a forced migration due to constant agitation.

Subsequent experiments providing quantitative data on the extent to which these inhibitors migrate from one phase to another will be presented later.

Disinfection of the Aqueous Phase

Materials and Methods

The water soluble disinfectants phenol, benzalkonium chloride, para-chloro-meta-cresol and Betadine (British Drug Houses) were used to study disinfection problems when microorganisms were suspended in the hydrocarbon phase and the disinfectant was added

to the aqueous phase.

The hydrocarbon phase (light liquid petrolatum) was inoculated with test bacteria by the lyophilization procedure when the amount of oil inoculum required was small. In experiments where large quantities of contaminated oil was required, cells were grown for 24 hours in T.C.S. broth, centrifuged and resuspended in phosphate buffer. Hydrocarbon was then mixed with the suspension for 48 hours on a mechanical wrist-action shaker. After shaking, the hydrocarbon layer was separated from the aqueous phase and several small aliquots placed in T.C.S. broth and incubated for 24 hours to assure that organisms had been suspended in the hydrocarbon.

Disinfectant Solutions

The aqueous disinfectant solutions were made up in the same manner as the hydrocarbon-disinfectant solutions used in the previous experiment, with the exception that enough disinfectant was dissolved in the aqueous phase to make the stated concentrations relative to the final volume.

The test conditions and methods of determining the presence of viable bacteria in the hydrocarbon and aqueous phases were the same as for the disinfectant in hydrocarbon system.

Results

TABLES XIV and XV show the effect of disinfectants on Staphylococcus aureus and Pseudomonas fluorescens when the test organisms are suspended in the hydrocarbon phase and the disinfectants are in the aqueous phase.

TABLE XIV

The Effect of Various Concentrations of Disinfectants
on Staphylococcus aureus F.D.A. 209 in
Hydrocarbon When the Disinfectant is in the Aqueous Phase

Disinfectant ^(A)	Phase Tested for Viable Cells							
	Oil Water		Oil Water		Oil Water		Oil Water	
Phenol	1%		0.5%		0.25%		0.12%	
	0	0	0	0	0	0	0	0
Benzalkonium Chloride	1:50,000		1:100,000		1:100,000		1:100,000	
	0	0	0	0	0	0	0	0
Para-Chloro-Meta-Cresol	1:2,000		1:3,000		1:3,500		1:4,000	
	0	0	0	0	0	0	0	0
Betadine (PVP-I) ^(C)	0.25%		0.125%		0.0625%		0.03%	
	0	0	0	0	0	0	0	0
Control ^(B)	+	+	+	+	+	+	+	+

A - Disinfectant concentrations are as indicated in the table.

B - The control consisted of mineral salts solution plus
contaminated hydrocarbon.

C - The concentration of Betadine is per cent available iodine.

+- Growth of the test organism.

0 - No growth of the test organism.

TABLE XV

The Effect of Various Concentrations of Disinfectants
on Pseudomonas fluorescens O.A.C. 99 in
Hydrocarbon When the Disinfectant is in the Aqueous Phase

Disinfectant (A)	Phase Tested for Viable Cells							
	Oil Water		Oil Water		Oil Water		Oil Water	
Phenol	1%		0.5%		0.25%		0.12%	
	0	0	0	0	0	0	0	0
Benzalkonium Chloride	1:50,000		1:100,000		1:100,000		1:110,000	
	0	0	+	+	+	+	+	+
Para-Chloro-Meta-Cresol	1:2,000		1:3,000		1:3,500		1:4,000	
	0	+	0	+	+	+	+	+
Betadine (PVP-I) (C)	0.25%		0.125%		0.0625%		0.03%	
	0	0	0	0	0	0	0	0
Control (B)	+	+	+	+	+	+	+	+

A - Disinfectant concentrations are as indicated in the table.

B - The control consisted of mineral salts solution plus
contaminated hydrocarbon.

C - The concentration of Betadine is per cent available iodine.

+- Growth of the test organism.

0 - No growth of the test organism.

Discussion of Results

Phenol

At a concentration of 0.12%, phenol is effective in killing both Staphylococcus aureus and Pseudomonas fluorescens in both the aqueous and the oil phases after 3 days of continuous exposure.

Benzalkonium Chloride

At a concentration of 1:50,000 benzalkonium chloride is effective against both test organisms in both phases of the test system. A 1:100,000 solution kills Staphylococcus in both phases but Pseudomonas fluorescens is unaffected in either phase.

Benzalkonium chloride was not expected to inhibit the test organisms in the oil phase since positively charged molecules (polar molecules) do not dissolve in a non-polar solvent such as light liquid petrolatum. However, since the mixture was shaken for 3 days, and the quaternary ammonium compounds can serve as emulsifying agents, it is conceivable that the formation of an emulsion and the presence of constant agitation and mixing resulted in direct contact of virtually all of the cells in the hydrocarbon with this disinfectant.

Para-Chloro-Meta-Cresol

A 1:2,000 concentration of this disinfectant is effective against Staphylococcus aureus and Pseudomonas fluorescens in both phases while a 1:3,000 dilution is effective against Staphylococcus and Pseudomonas in the oil phase and Staphylococcus in the aqueous phase but not against Pseudomonas in the aqueous phase. A 1:3,500 concentration is effective against Staphylococcus in both phases

but is not effective against Pseudomonas in either phase. A 1:4,000 solution is ineffective against either organism in either phase of the mixture.

Betadine (PVP-I)

Betadine is effective in both the aqueous and the hydrocarbon phases for Staphylococcus and Pseudomonas at all concentrations investigated. Kohn et al. (1963), in comparing the efficiency of antibacterial agents against Pseudomonas aeruginosa found that 100, 50 and 25 p.p.m. of iodine would kill his strain of Pseudomonas in 15 minutes. Our test time was 72 hours therefore, it is likely that concentrations much lower than those we tested would have inhibited the organisms.

It must be remembered that with all these disinfectant systems there are two migration processes occurring simultaneously.

- 1) The organisms are migrating from the hydrocarbon to the aqueous phase.
- 2) Some of the disinfectant is partitioning into the hydrocarbon phase from the aqueous phase.

Studies on the Dynamics of Disinfection of Oil-Water Mixtures

The use of a spectrophotometric method in the quantitation of phenolic compounds in pharmaceutical preparations has been described by Elridge and Pentrell (1961). Their results indicated that quantitative determinations of a variety of phenols in a wide variety of pharmaceutical preparations and cosmetics, gave good agreement with the chemical analyses which were available.

For our investigation a spectrophotometric procedure was employed to show the actual quantitative partitioning (distribution coefficient) of various antimicrobial agents employed in the

disinfection of oil and water combinations.

The results of our previous experiments in which the test bacteria were placed in the hydrocarbon phase and the disinfectant in the aqueous or in which the bacteria were placed in the aqueous phase and the disinfectant in the hydrocarbon phase indicate that two migrations are occurring simultaneously under our test conditions.

- 1) Bacteria migrate from the aqueous phase to the hydrocarbon phase and vice-versa in oil-water mixtures.
- 2) Migration of certain disinfectants from the aqueous phase to the hydrocarbon phase and vice-versa in oil-water mixtures occurs.

We now attempted to investigate the migration of the disinfectants in greater detail.

Absorption Maxima

It was first necessary to determine the absorption maximum for each of the inhibitors being studied. This was done by preparing solutions of the disinfectants in deionized water and then scanning these solutions using a Spectronic 505 Recording Spectrophotometer.

GRAPH V and TABLE XVI indicate the absorption characteristics for aqueous solutions of the disinfectants being studied.

GRAPH V

ABSORPTION SPECTRA FOR AQUEOUS SOLUTIONS OF FIVE DISINFECTANTS

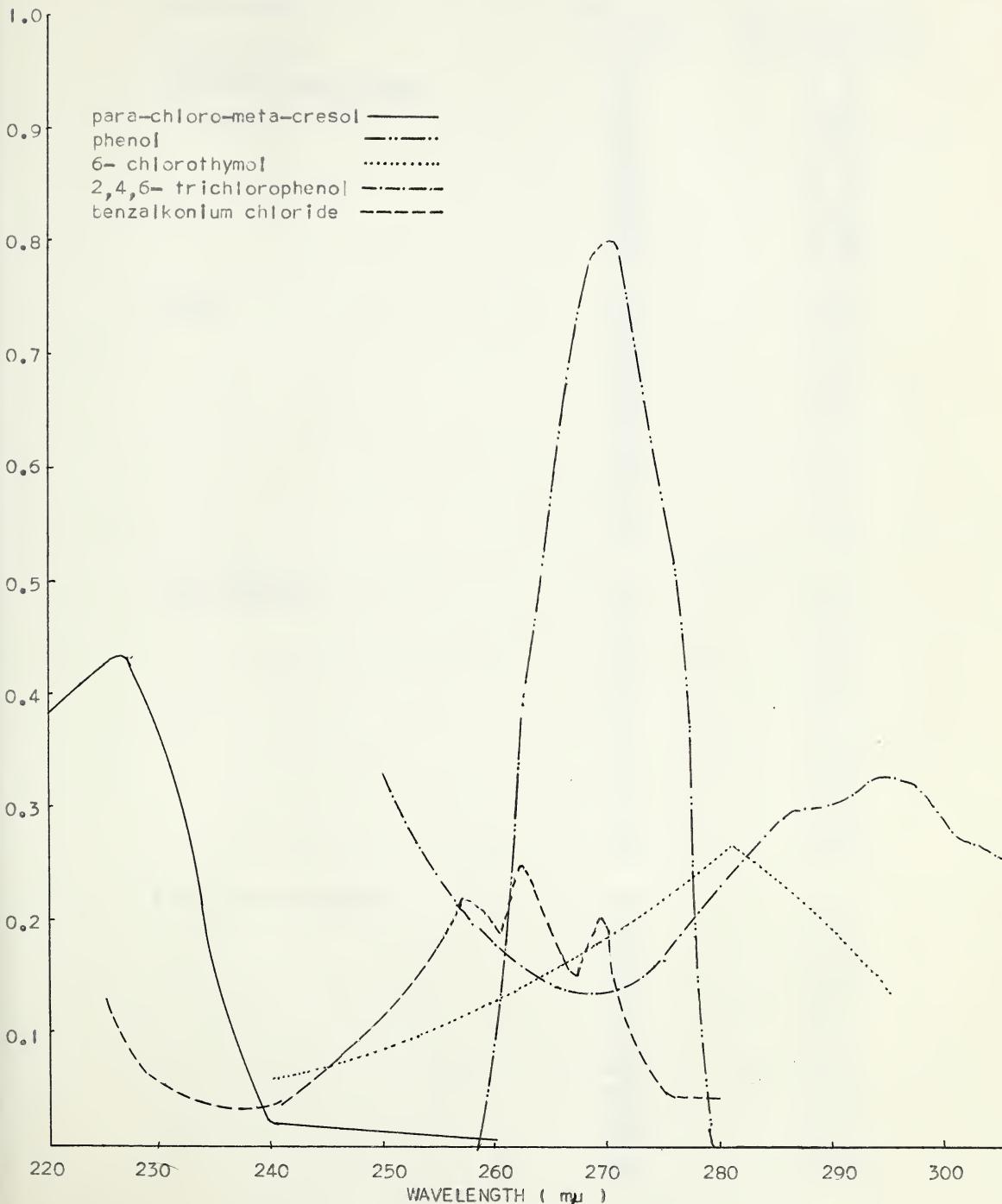


TABLE XVI

Absorption Spectra Data for Aqueous Solutions
of 5 Disinfectants

Disinfectant	Wavelength (m μ)	Optical Density (O.D.)
Para-chloro-meta-cresol	220	0.38
	225	0.425
	227	0.43
	230	0.375
	235	0.15
	240	0.02
	250	0.005
	260	0.005
Phenol	259	0.00
	260	0.19
	262	0.38
	265	0.55
	266	0.65
	270	0.80
	271	0.75
	275	0.55
	277	0.35
	278	0.15
	279	0.00
6-chlorothymol	240	0.06
	250	0.09
	260	0.13
	265	0.16
	270	0.19
	275	0.23
	280	0.26
	281	0.265
	282	0.26
	285	0.245
	290	0.19
	295	0.135
2,4,6-trichlorophenol	250	0.33
	255	0.24
	260	0.18
	265	0.145
	270	0.14
	275	0.17
	278	0.21
	285	0.29
	290	0.305
	295	0.33
	300	0.28
	305	0.255

TABLE XVI (Cont)

Absorption Spectra Data for Aqueous Solutions
of 5 Disinfectants

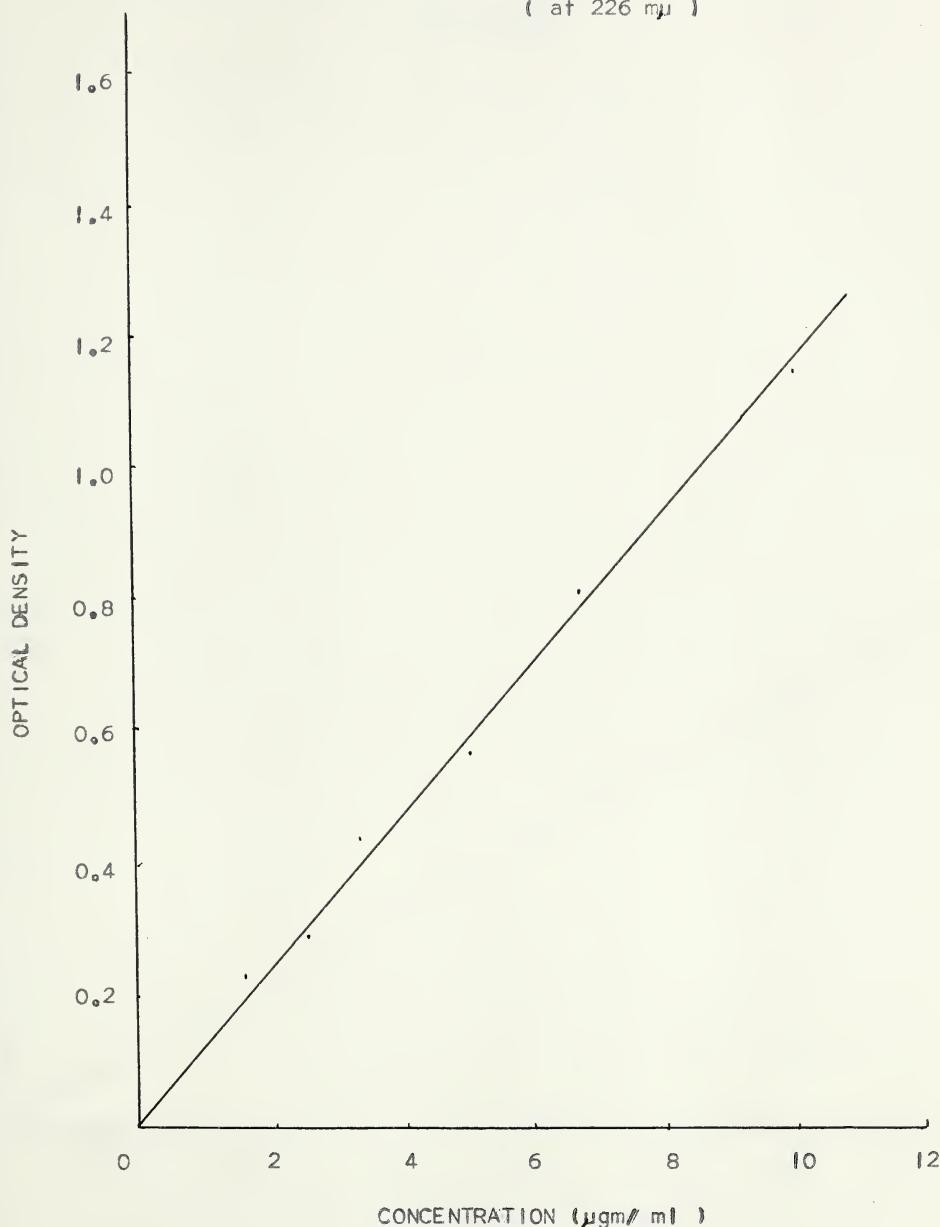
<u>Disinfectant</u>	<u>Wavelength (mμ)</u>	<u>Optical Density (O.D.)</u>
Benzalkonium chloride	225	0.13
	230	0.05
	235	0.035
	240	0.035
	245	0.065
	250	0.12
	255	0.18
	257	0.215
	260	0.19
	262	0.25
	265	0.18
	267	0.15
	269	0.20
	270	0.14
	275	0.045
	280	0.045

Absorption maxima used for subsequent experiments (from TABLE XVI and GRAPH V) are:

- 1) 2,4,6-trichlorophenol 285 m μ
- 2) 6-chlorothymol 281 m μ
- 3) Phenol 270 m μ
- 4) Benzalkonium chloride 262 m μ
- 5) Para-chloro-meta-cresol 226 m μ

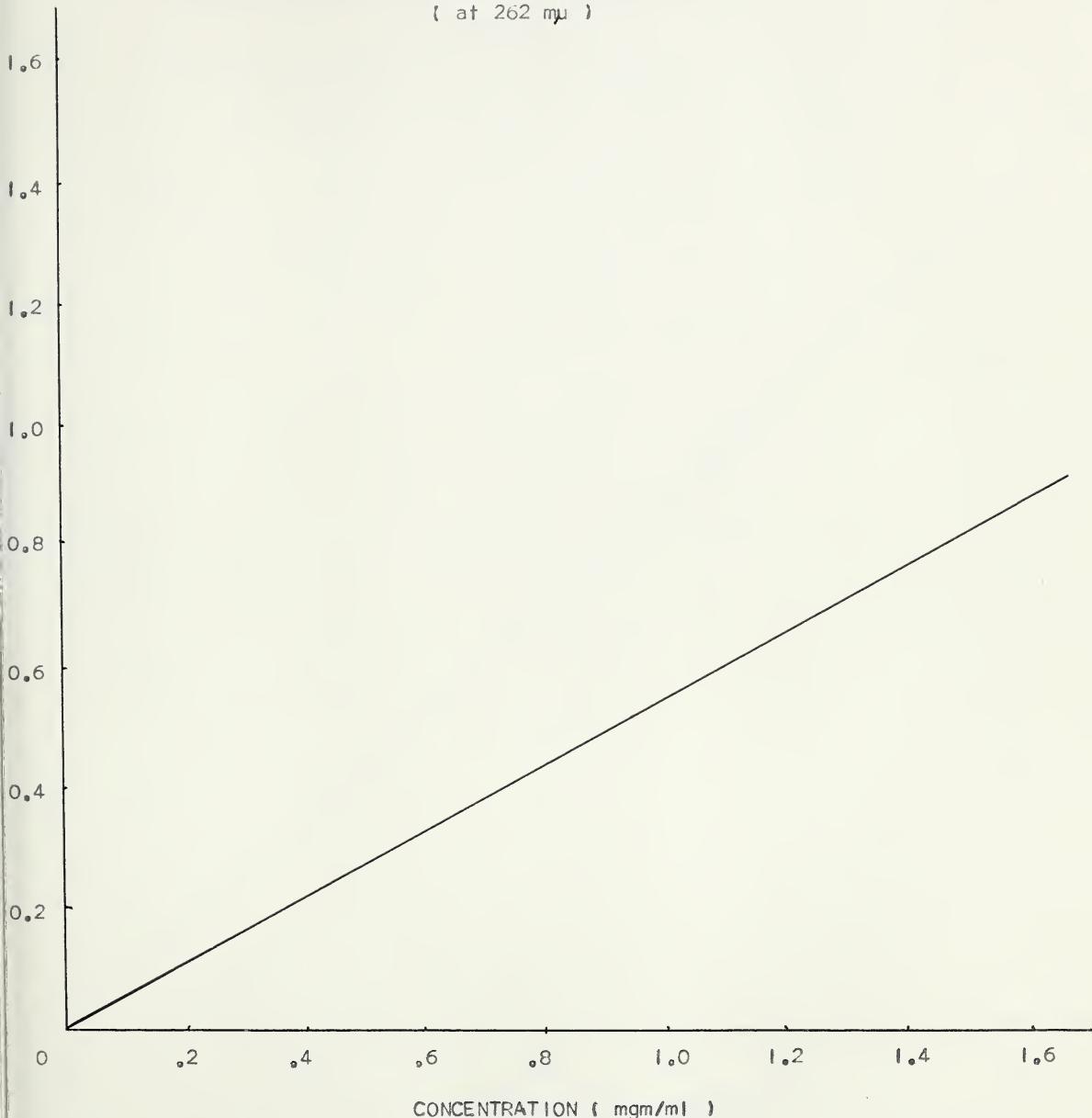
GRAPH VI

STANDARD CURVE FOR PARA-CHLORO-META-CRESOL IN AQUEOUS SOLUTION

(at 226 μ)

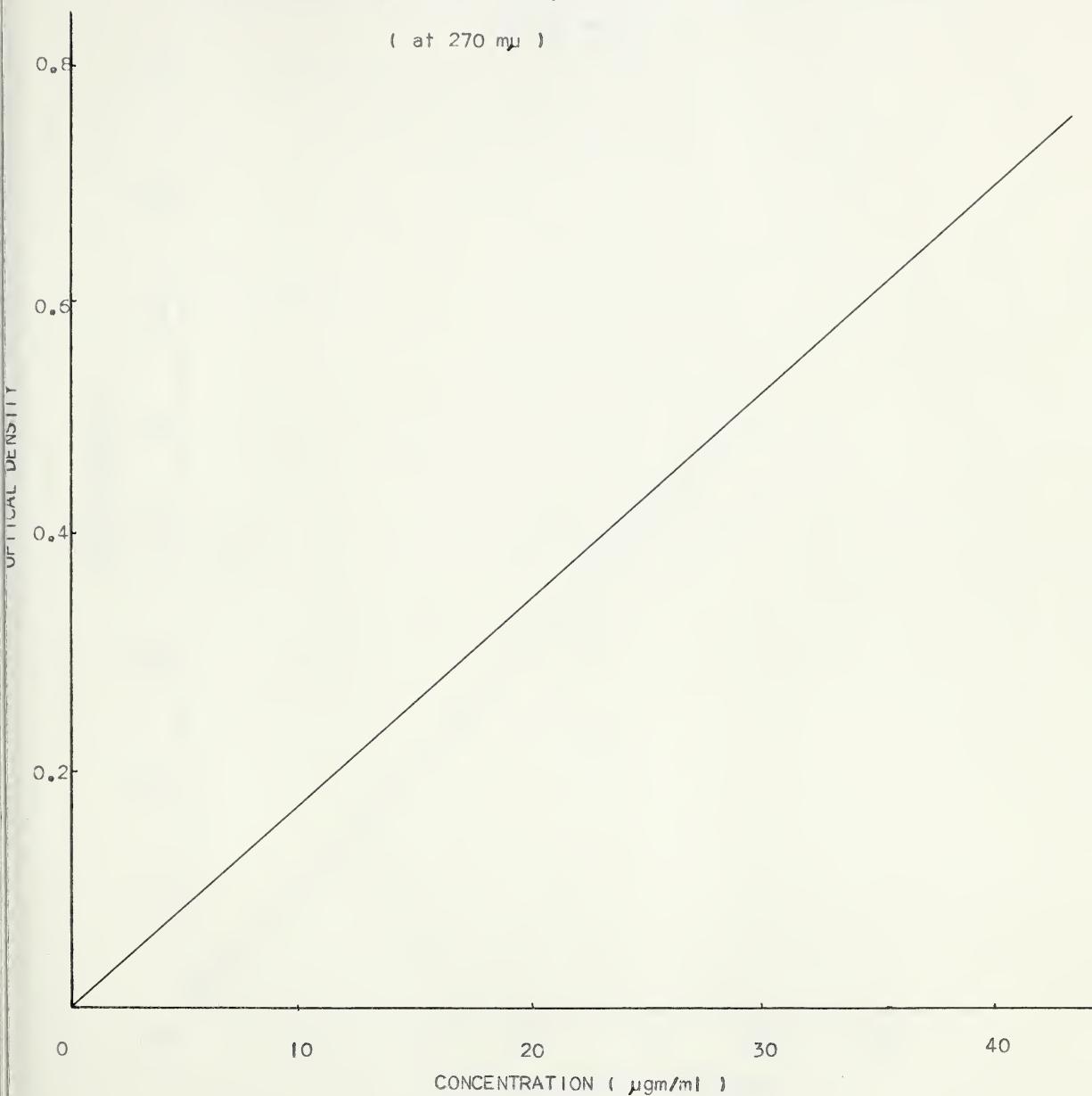
GRAPH VII

STANDARD CURVE FOR BENZALKONIUM CHLORIDE IN AQUEOUS SOLUTION

(at 262 μ)

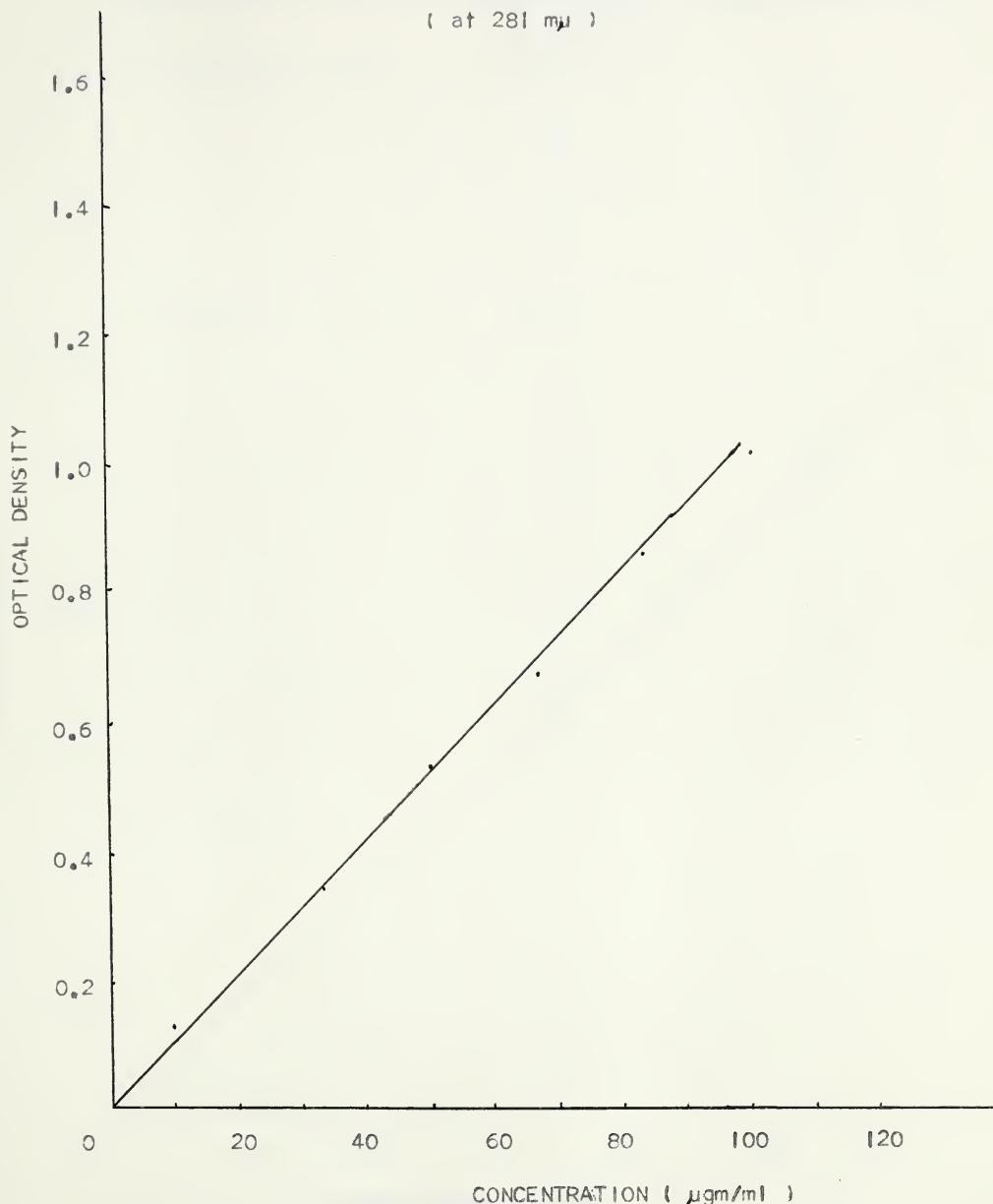
GRAPH VIII

STANDARD CURVE FOR PHENOL IN AQUEOUS SOLUTION

(at 270 μ)

GRAPH IX

STANDARD CURVE FOR 6- CHLOROTHYMOl IN AQUEOUS SOLUTION



GRAPH X

STANDARD CURVE FOR 2,4,6-TRICHLOROPHENOL IN AQUEOUS SOLUTION

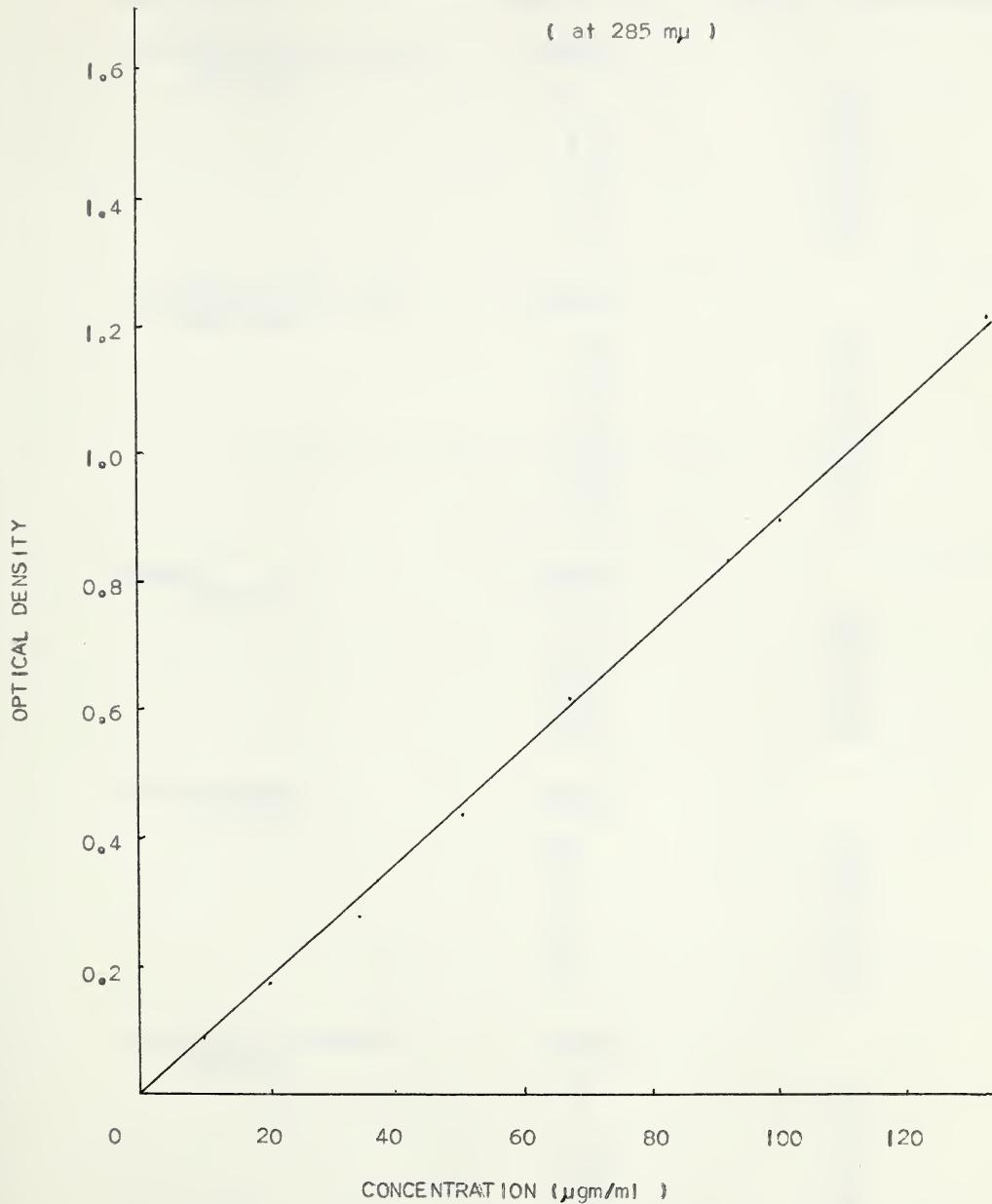


TABLE XVIIPreparation of Standard Curves for Disinfectants
in Aqueous Solution

<u>Disinfectant</u>	<u>Concentration</u>	<u>Optical Density (O.D.)</u>
Para-chloro-meta-cresol (226 $\mu\mu$)	<u>$\mu\text{gm}/\text{ml}$</u>	
10		1.14
6.7		0.80
5.0		0.56
3.3		0.43
2.5		0.29
1.6		0.24
Benzalkonium chloride (262 $\mu\mu$)	<u>mgm/ml</u>	
1.67		0.90
1.25		0.72
1.0		0.54
0.63		0.38
0.42		0.23
0.20		0.13
0.10		0.08
Phenol (270 $\mu\mu$)	<u>$\mu\text{gm}/\text{ml}$</u>	
4.5		0.08
11.3		0.20
22.5		0.40
30.0		0.52
45.0		0.80
6-chlorothymol (281 $\mu\mu$)	<u>$\mu\text{gm}/\text{ml}$</u>	
100		1.01
83		0.86
67		0.67
50		0.53
33		0.34
10		0.13
2,4,6-trichlorophenol (285 $\mu\mu$)	<u>$\mu\text{gm}/\text{ml}$</u>	
10		0.09
20		0.17
33.5		0.28
50.0		0.43
67.0		0.62
100.0		0.89
133.0		1.21

Preparation of Standard Curves for Disinfectants in Water.

After determining a reference wavelength for each disinfectant it was necessary to prepare standard curves of optical density versus disinfectant concentration.

Disinfectant solutions, of varying concentrations, were prepared in deionized water. Optical densities were recorded in a Spectronic 505 Recording Spectrophotometer using deionized water as the zeroing blank solution.

The standard curves and data used to prepare them are presented in GRAPHS VI to X and TABLE XVII.

Distribution Studies on Disinfectants in ShakingOil-Water Mixtures

To study the distribution coefficient of a particular inhibitor, a mixture of 10% hydrocarbon in aqueous inhibitor solution was prepared. This mixture was shaken for 20 hours at a setting of "5" on the Burrell Wrist-Action Shaker scale. The concentration of inhibitor in the aqueous phase was then measured spectrophotometrically and compared to the concentration of a control solution which had not been in contact with light liquid petrolatum.

According to Bentley's Textbook of Pharmaceutics, (Davis, 1956) the distribution coefficient of a substance is the distribution of a substance between two non-miscible solvents, A and B, where

$$\frac{\text{Concentration in A}}{\text{Concentration in B}} = \text{the distribution coefficient}$$

or briefly $\frac{C_A}{C_B} = K$

Since saturated solutions of the same solute in two immiscible solvents are in equilibrium, the distribution coefficient or partition coefficient is equal to the ratio of the solubility of the solute in solvent A to it's solubility in solvent B. In most cases however, K is not strictly constant for all ranges of concentrations in the two solvents. The value of K derived from the solubilities is therefore only approximate.

Because the solutions we studied were not saturated solutions and therefore exact coefficients cannot be derived, we shall report our observations in terms of per cent migration of the disinfectant from one phase into the opposite phase.

The results of our experiments are reported in TABLES XVIII and XIX.

Results

TABLE XVIII

Concentration of Control Disinfectant Solutions

<u>Disinfectant</u>	<u>Optical Density</u>	<u>Concentration</u>	<u>Dilution Factor</u>	<u>Actual Concentration</u>
6-chlorothymol	0.19	19 $\mu\text{gm}/\text{ml}$	10	190 $\mu\text{gm}/\text{ml}$
Phenol	0.40	22.5 $\mu\text{gm}/\text{ml}$	2	45 $\mu\text{gm}/\text{ml}$
Para-chloro-meta-cresol	0.495	4.25 $\mu\text{gm}/\text{ml}$	100	425 $\mu\text{gm}/\text{ml}$
Benzalkonium chloride		Cannot be used for this Experiment		
2,4,6-trichlorophenol	0.18	20 $\mu\text{gm}/\text{ml}$	10	200 $\mu\text{gm}/\text{ml}$

TABLE XIX

Migration of Disinfectant From An Aqueous to a Hydrocarbon Phase after Shaking for 20 hours

Disinfectant	Optical Density	Concen- tration μgm/ml	Dilu- tion	Final Concen- tration μgm/ml	Initial Concen- tration μgm/ml	% Migration
6-chlorothymol	0.02	2	10	20	190	89.5
Phenol	0.36	20.5	2	41	45	9
Para-chloro-meta-cresol	0.25	2.0	100	200	425	52.9
Benzalkonium chloride			Cannot be used for this Experiment			
2,4,6-trichlorophenol	0.10	12	10	120	200	40

Discussion

The aqueous solution of benzalkonium chloride, when shaken with light liquid petrolatum, forms a very fine emulsion. This is to be expected since quaternary ammonium compounds are surface-active agents and act as cationic emulsifying agents, forming oil in water emulsions. An attempt was made to break the emulsion by quickly freezing and thawing but this was not successful in resolving the system into distinct phases.

From the results obtained using shaking mixtures, containing 10% oil phase and 90% aqueous disinfectant, it was noted that the greatest migration occurred with 6-chlorothymol (89.5%), then para-chloro-meta-cresol (52.9%), 2,4,6-trichlorophenol (40%) and the least amount of migration occurred with phenol (9%).

Distribution Studies on Disinfectants in Stationary

Oil-Water Mixtures

Using the standard para-chloro-meta-cresol, 2,4,6-trichlorophenol, phenol and 6-chlorothymol solutions prepared previously, the distribution or partitioning effect of these disinfectants was studied on stationary mixtures using two different concentrations

of hydrocarbon. i.e. 10% hydrocarbon and 25% hydrocarbon.

Light liquid petrolatum was carefully layered on the surface of the standard disinfectant solutions so as to prevent mixing of the two phases as much as possible.

The two test systems were set up so that the 10% hydrocarbon system contained 9 ml of disinfectant solution and 1 ml of hydrocarbon and the 25% hydrocarbon system contained 7.5 ml of disinfectant solution and 2.5 ml of hydrocarbon.

The mixtures were contained in 50 ml Erlenmeyer flasks at all times so that the oil-water interfacial area would be relatively constant for all tests. The flasks were maintained at room-temperature for this series of experiments.

Controls which contained no hydrocarbon were treated in exactly the same manner in so far as temperature, vessel size, amount of handling and exposure to light is concerned.

Contact time between phases was 48 hours. After 48 hours the hydrocarbon phase (top layer) was removed and the aqueous bottom shaken to insure uniform distribution of the remaining disinfectant throughout the water. A sample was removed, dilutions made if necessary, and the optical density was measured at a predetermined wavelength. The values thus obtained were then applied to the standard curves and concentrations read off.

Values for the standards and the test solutions were compared.

The percent migration was determined using the control as zero migration and calculations made thus:

e.g. Migration of 6-chlorothymol in 10% hydrocarbon.

190 - 15 = 175 $\mu\text{gm}/\text{ml}$ migrated or

$$\frac{175}{190} \times 100 = 92.1\%$$

Results

The effect of hydrocarbon concentration on migration is shown in the following table.

TABLE XX

The Effect of 10% and 25% Oil on the Migration of Disinfectants from an Aqueous to Oil Phase when the Mixtures are Stationary for 48 hours

<u>Disinfectant</u>		<u>Optical Sample*</u>	<u>Density</u>	<u>Dilution</u>	<u>Concen- tration</u> ($\mu\text{gm}/\text{ml}$)	<u>% Migra- tion</u>
6-chlorothymol	Control	0.195	10	190	-	
	10%H.C.	0.015	10	15	92.1	
	25%H.C.	0.045	0	5	97.3	
2,4,6-trichlorophenol	Control	0.20	10	230	-	
	10%H.C.	0.06	10	67	70.8	
	25%H.C.	0.055	10	62	73.0	
P-C1-M-cresol	Control	0.565	100	480	-	
	10%H.C.	0.485	100	400	16.7	
	25%H.C.	0.370	100	310	35.4	
Phenol	Control	0.43	2	49	-	
	10%H.C.	0.415	2	47	4	
	25%H.C.	0.405	2	46	6.1	

* H.C. - Hydrocarbon

— - no reading taken

Discussion

In each instance, with the four inhibitors investigated, the migration into the hydrocarbon phase was greater where the hydrocarbon concentration was 25%. This phenomenon has many industrial implications especially where phenol and derivatives thereof are

involved since these compounds are active due to their ability to migrate rapidly from the aqueous to the lipoidal phase as represented by the microbial cell (Judis, 1964).

The presence of hydrocarbon such as the light liquid petrolatum used in our experiments represents an additional lipid phase resulting in reduction of the effective disinfectant concentration in the aqueous bottom layer.

If an oil and water system were set up where the ratio of oil to water was known and constant, the amount of a particular inhibitor necessary to disinfect the system could be calculated. If, after this system had been effectively disinfected, the amount of oil were suddenly increased, an additional loss of phenolic inhibitor from the aqueous phase would result. Now the concentration of the disinfectant located in the aqueous phase is ineffective and more inhibitor must be added to compensate for migration.

Our observations are in agreement with those of Bennett (1963) who says that emulsions constitute additional lipoidal phases and there is considerable reduction in environmental concentrations of disinfectant because of this effect.

Migration of Disinfectant from Hydrocarbon to
an Aqueous Phase

Materials and Method

To measure the migration of a disinfectant from a hydrocarbon phase to an aqueous phase, a known volume of hydrocarbon containing a known concentration of disinfectant was added to a known volume of deionized water in a 125 ml Erlenmeyer flask. The mixture was allowed to stand for 48 hours at room-temperature (22 - 25°C).

The amount of disinfectant migrating into the aqueous phase in a given length of time (48 hours) was determined spectrophotometrically. The hydrocarbon concentrations used were 10% and 25% v/v . The control contained hydrocarbon and water but no disinfectant.

The following disinfectant-hydrocarbon solutions were studied:

- a) Phenol 1% w/v
- b) 2,4,6-trichlorophenol 1% w/v
- c) 6-chlorothymol 1% w/v
- d) Para-chloro-meta-cresol 0.5% w/v

Results

The method used to calculate per cent migration of disinfectant from a hydrocarbon phase to an aqueous phase is as follows:

e.g. 1% Phenol in hydrocarbon \equiv 10,000 g/ml

$$\% \text{ migration} = \frac{\text{Amount of disinfectant in aqueous phase}}{\text{Amount of disinfectant in hydrocarbon}}$$

Therefore in the 10% hydrocarbon sample the amount of migration $= \frac{950}{10,000} \times 100 = 9.5\%$ into the aqueous phase

for the 25% hydrocarbon sample the amount of

migration $= \frac{2,650}{10,000} \times 100 = 26.5\%$ into the aqueous phase.

The results of this experiment are shown in the following table.

TABLE XXI

The Migration of Disinfectants From an Oil to an
Aqueous Phase of an Oil-Water Mixture

Disinfectant	% Hydro-carbon in Mixture	O.D. of Aqueous Phase	Final Concen- tration* (μ gm/ml)	% Migration
Phenol (at 270 $\text{m}\mu$)	10%	0.17	950	9.5
	25%	0.47	2650	26.5
2,4,6-trichlorophenol (at 285 $\text{m}\mu$)	10%	0.94	104	1.04
	25%	0.86	96	0.96
6-chlorothymol (at 281 $\text{m}\mu$)	10%	0.68	65	0.65
	25%	0.73	70	0.70
P-C1-M-cresol (at 226 $\text{m}\mu$)	10%	0.265	220	2.2
	25%	0.60	510	5.1

* The final concentrations for phenol and para-chloro-meta-cresol are 100 times more than the optical density values indicate since 1 in 100 dilutions of the aqueous phase were made at the time of spectrophotometric reading.

Discussion of Results

When the four test disinfectants were dissolved in water and their migration into hydrocarbon phases measured, it was seen that 6-chlorothymol had the greatest affinity for the oil phase followed by 2,4,6-trichlorophenol, para-chloro-meta-cresol and phenol.

(TABLE XX) When the same disinfectants were dissolved in hydrocarbon and their migration into aqueous phases measured (TABLE XXI) it was seen that the disinfectants which had exhibited the strongest affinity for the hydrocarbon phase when dissolved in water exhibited the least amount of migration from oil to water when dissolved in oil.

Migration of Disinfectant From Aqueous to
Hydrocarbon Phase as a Function of Time

The four disinfectants used in previous experiments were also used when the influence of time on migration was studied. The total volume of the hydrocarbon-water mixture was 50 ml of which 10% was hydrocarbon phase. The mixtures were kept stationary at room-temperature (22 - 25°C) in 125 ml Erlenmeyer flasks.

Method

Several replicates of each mixture of hydrocarbon-water and disinfectant were prepared so that a replicate could be used for each reading thus eliminating the possible effect of agitation when samples are removed from a single large volume of mixture.

At various time intervals the aqueous phase of the hydrocarbon-water and disinfectant mixture was removed and the optical density determined on a Spectronic 505 Recording Spectrophotometer. The values obtained were compared to values for a control disinfectant solution which had not been in contact with hydrocarbon.

Results

The data from these experiments was plotted in two ways. The first graph compared optical density versus time and the second concentration versus time, however, a ΔD_c was also calculated where

ΔD_c = difference between the concentration of the disinfectant in the control solution and the concentration in the test solution at similar time intervals. The noted difference being due to migration of disinfectant from the aqueous phase into the hydrocarbon phase.

That is, concentration in control-concentration in test = amount of migration

or mathematically

$$\Delta D_c = C_c - C_t$$

The following tables and graphs demonstrate the effect of time on the migration of disinfectants from aqueous to hydrocarbon phases of oil-water mixtures.

TABLE XXII

The Effect of Time on the Migration of Disinfectants from Aqueous to Hydrocarbon Phases in Oil-Water Mixtures

Disinfectant	Time (Hours)	Optical Density		Concentration		ΔD_c ($\mu\text{gm}/\text{ml}$)
		Control	Test	Control	Test	
<i>2,4,6-trichlorophenol</i>						
(A)	0	1.66	-	186	-	-
	1	1.63	0.91	182	101	81
	4	1.58	0.29	176	33	143
	8	1.58	0.14	176	16	160
	14	1.57	0.125	175	14	161
	24	1.57	0.115	175	13	162
	30	1.58	0.13	176	15	162
	48	1.57	0.14	175	16	159
	55	1.58	0.12	176	14	162
<i>2,4,6-trichlorophenol</i>						
(B)	0	1.66	-	186	-	-
	1	1.63	0.84	182	98	89
	4	1.58	0.28	176	32	144
	8	1.58	0.145	176	16.5	159.5
	14	1.57	0.115	175	13	162
	24	1.57	0.085	175	10	165
	30	1.58	0.115	176	13	163
	48	1.57	0.12	175	14	161
	55	1.58	0.11	176	13	163

TABLE XXII

The Effect of Time on the Migration of
Disinfectants from Aqueous to Hydrocarbon
Phases in Oil-Water Mixtures

Disinfectant	Time (Hours)	Optical Density		Concentration		ΔD_c ($\mu\text{gm}/\text{ml}$)
		Control	Test	Control	Test	
($\mu\text{gm}/\text{ml}$)						
Phenol	0	0.45	-	25.5	-	-
	1	0.435	0.42	24.75	24.0	0.75
	4	0.445	0.43	25.25	24.5	0.75
	8	0.42	0.43	24	24.5	0.5
	12	0.46	0.45	26	25.5	0.5
	24	0.455	0.435	25.75	24.75	1.0
	30	0.46	0.45	26	25.5	0.5
	48	0.46	0.44	26	25	1.0
	72	0.45	0.44	25.5	25	0.5
6-chlorothymol	0	0.215	-	205	-	-
	1	0.20	0.07	195	70	125.0
	4	0.185	0.105	180	10	170.0
	8	0.195	0.095	190	9.5	180.5
	12	0.195	0.11	190	11	179.0
	24	0.195	0.08	190	8.0	182.0
	30	0.20	0.105	195	10.0	185.0
	48	0.20	0.13	195	13.0	182.0
	72	0.195	0.115	190	11.5	178.5

The control was diluted 10 fold for each reading.

P-Cl-M-cresol	0	0.53	-	450	-	450
	1	0.53	0.52	450	440	10
	4	0.55	0.485	470	405	65
	8	0.56	0.465	480	400	80
	12	0.55	0.47	470	400	70
	24	0.565	0.485	482	405	77
	30	0.565	0.480	482	402	80
	48	0.55	0.480	470	402	68

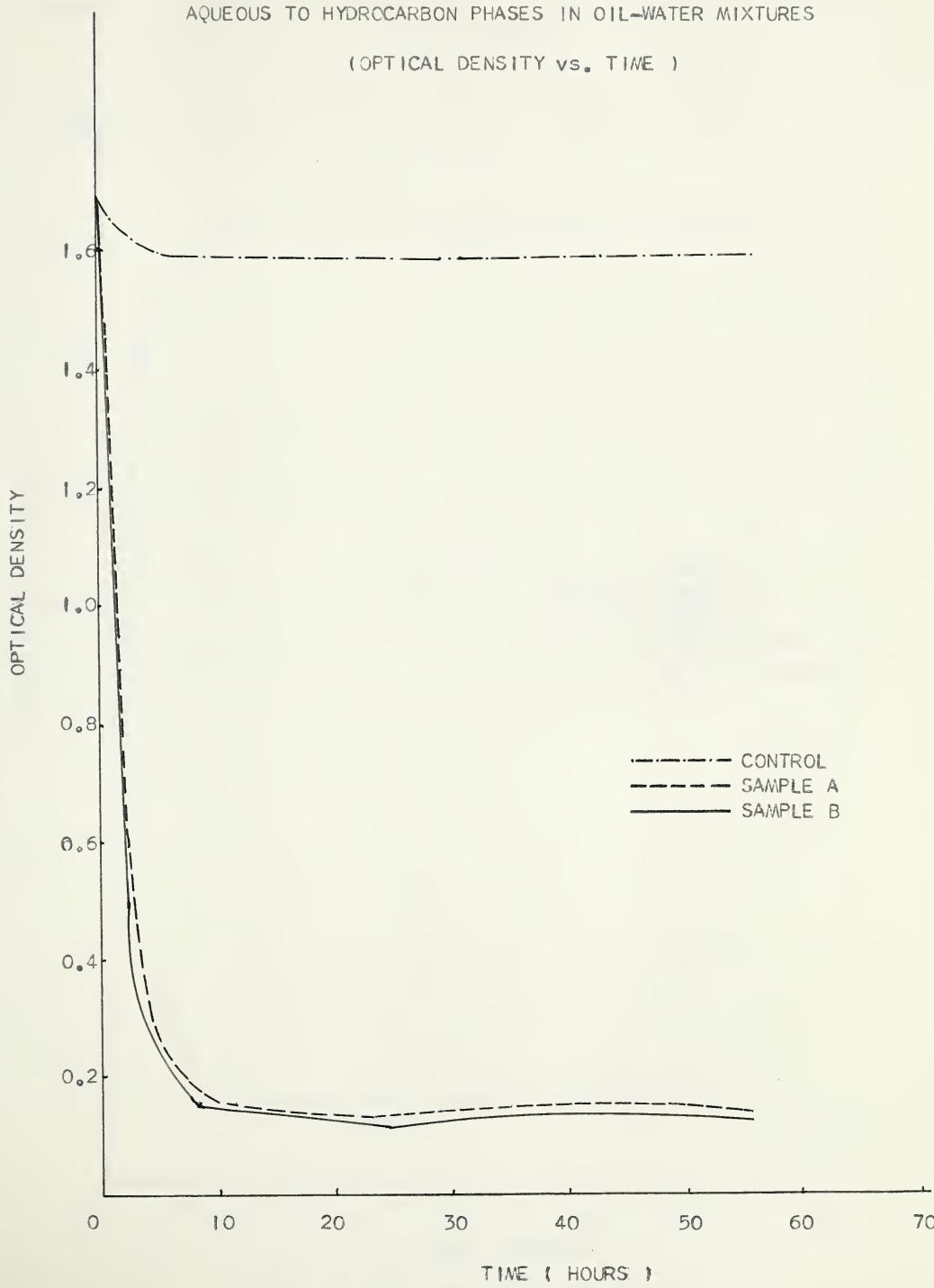
Test and Control solutions diluted 100 fold for each reading.

GRAPH XI

THE EFFECT OF TIME ON THE MIGRATION OF 2,4,6-TRICHLOROPHENOL FROM

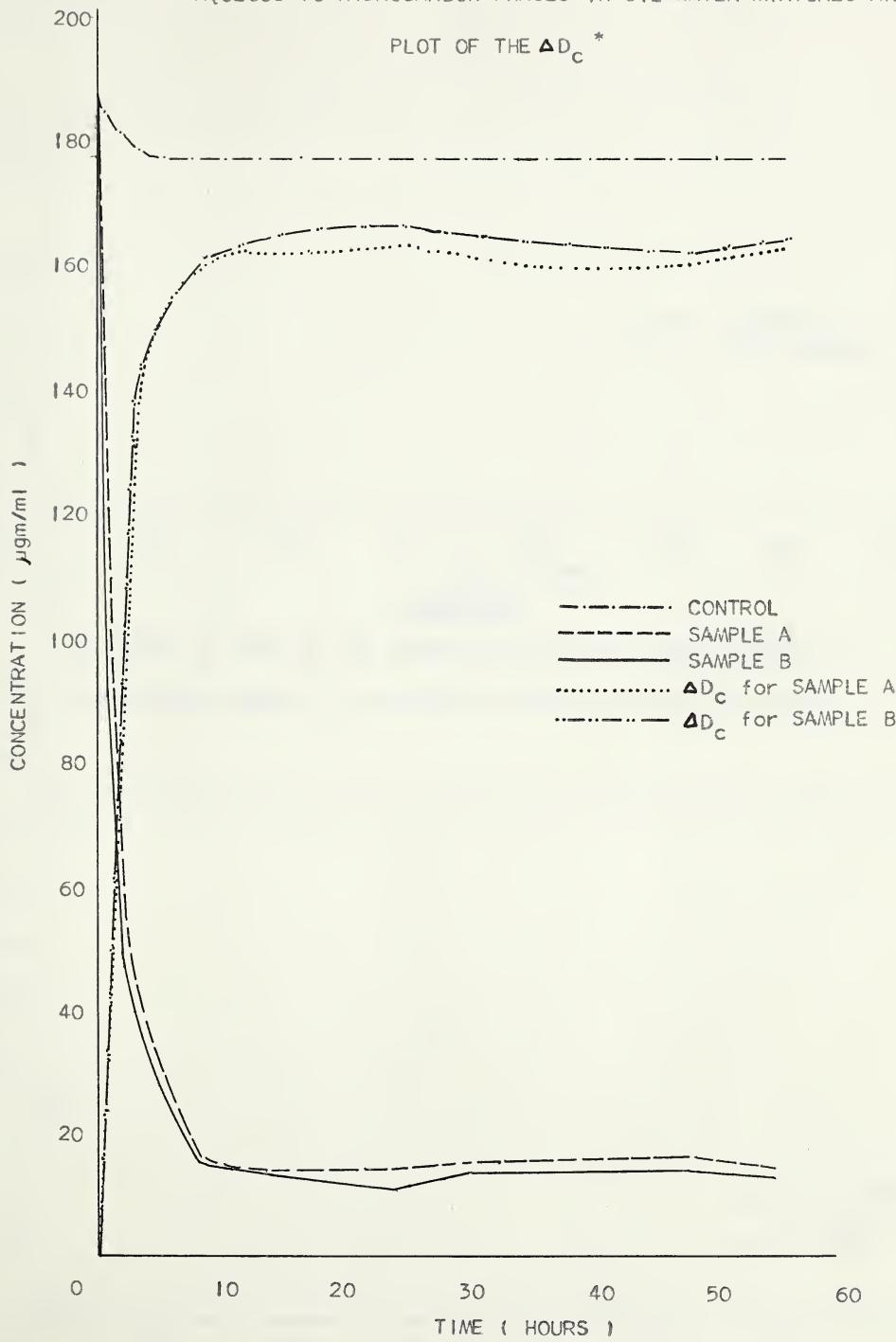
AQUEOUS TO HYDROCARBON PHASES IN OIL-WATER MIXTURES

(OPTICAL DENSITY vs. TIME)



GRAPH XII

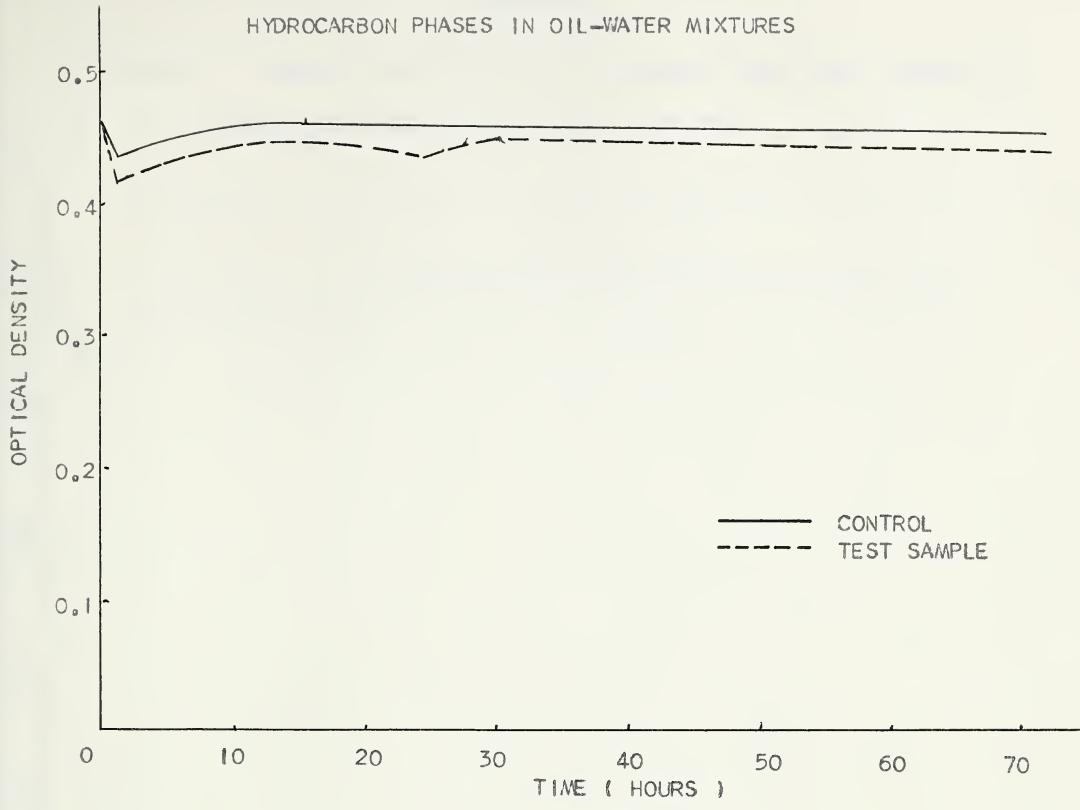
THE EFFECT OF TIME ON THE MIGRATION OF 2,4,6-TRICHLOROPHENOL FROM
AQUEOUS TO HYDROCARBON PHASES IN OIL-WATER MIXTURES AND



* Change in disinfectant concentration

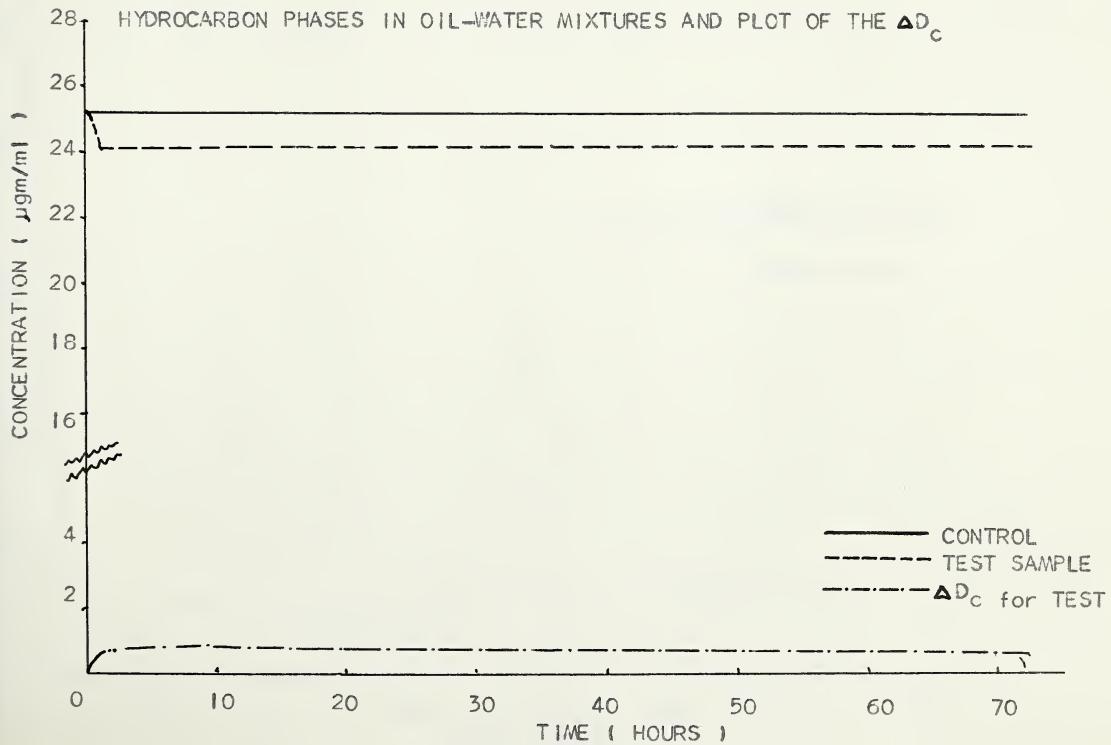
THE EFFECT OF TIME ON THE MIGRATION OF PHENOL FROM AQUEOUS TO

HYDROCARBON PHASES IN OIL-WATER MIXTURES



GRAPH XIV

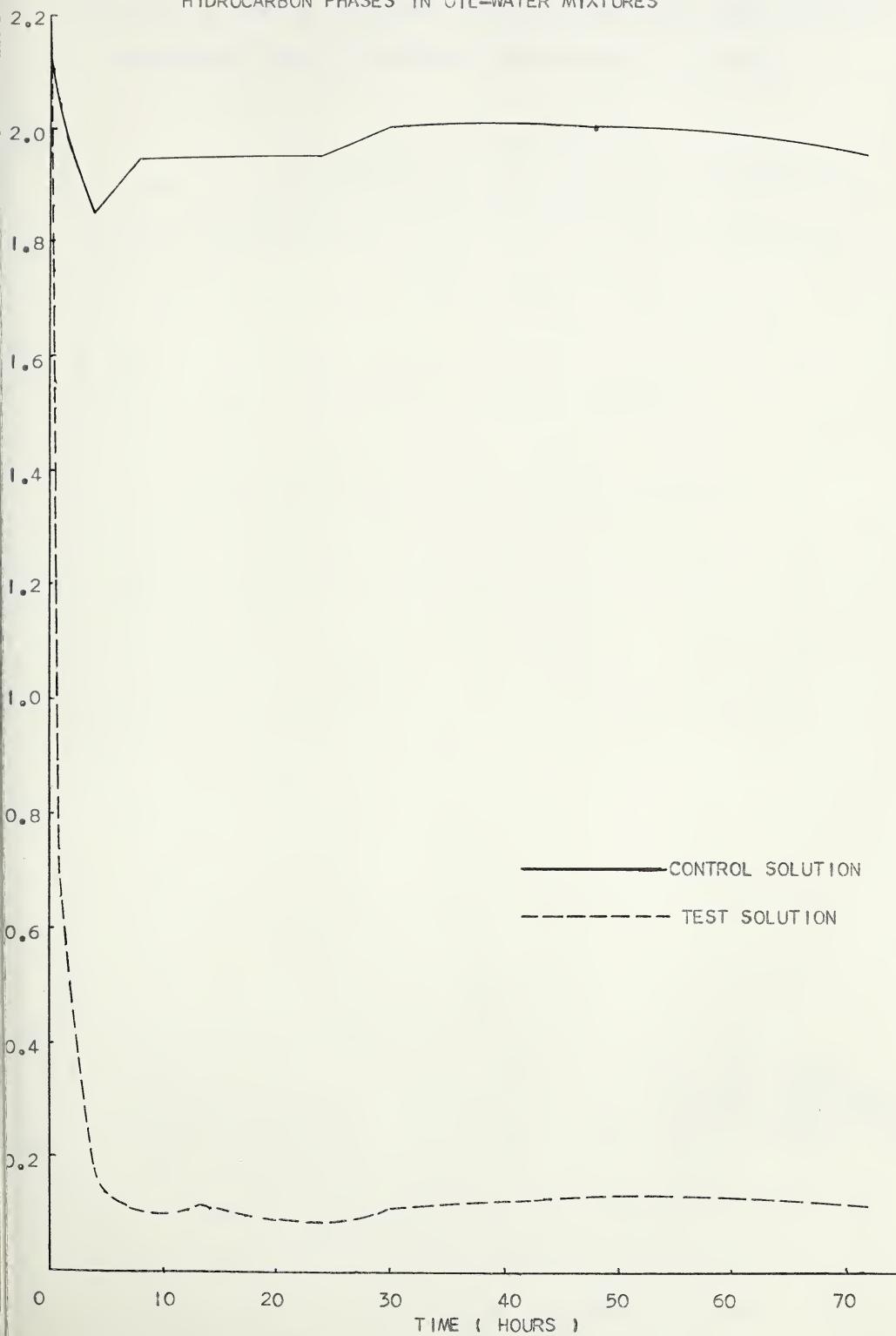
THE EFFECT OF TIME ON THE MIGRATION OF PHENOL FROM AQUEOUS TO



GRAPH XV

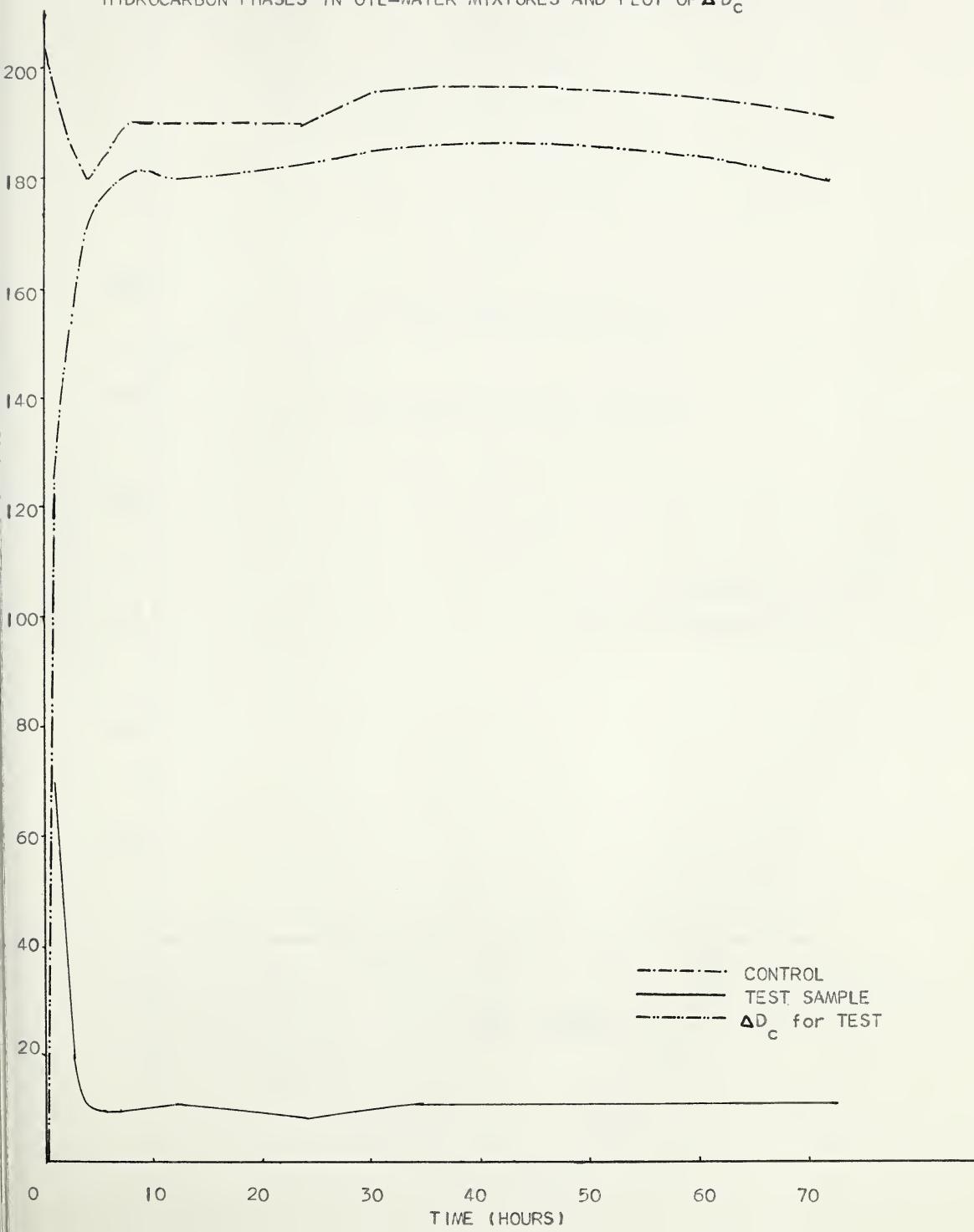
THE EFFECT OF TIME ON THE MIGRATION OF 6-CHLOROTHYMPHOL FROM AQUEOUS TO

HYDROCARBON PHASES IN OIL-WATER MIXTURES



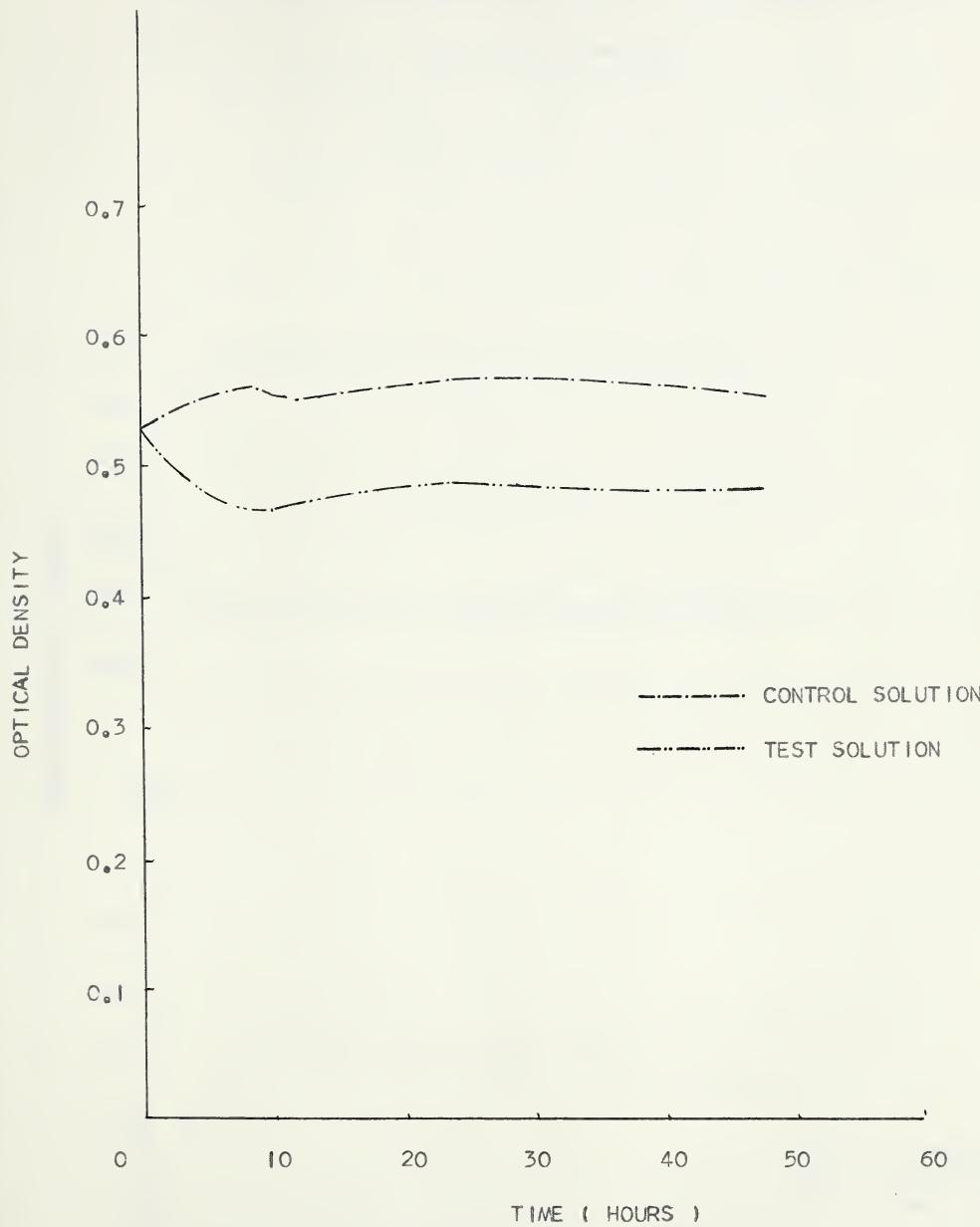
GRAPH XVI

THE EFFECT OF TIME ON THE MIGRATION OF 6-CHLOROTHYMOl FROM AQUEOUS TO
HYDROCARBON PHASES IN OIL-WATER MIXTURES AND PLOT OF ΔD_c



GRAPH XVII

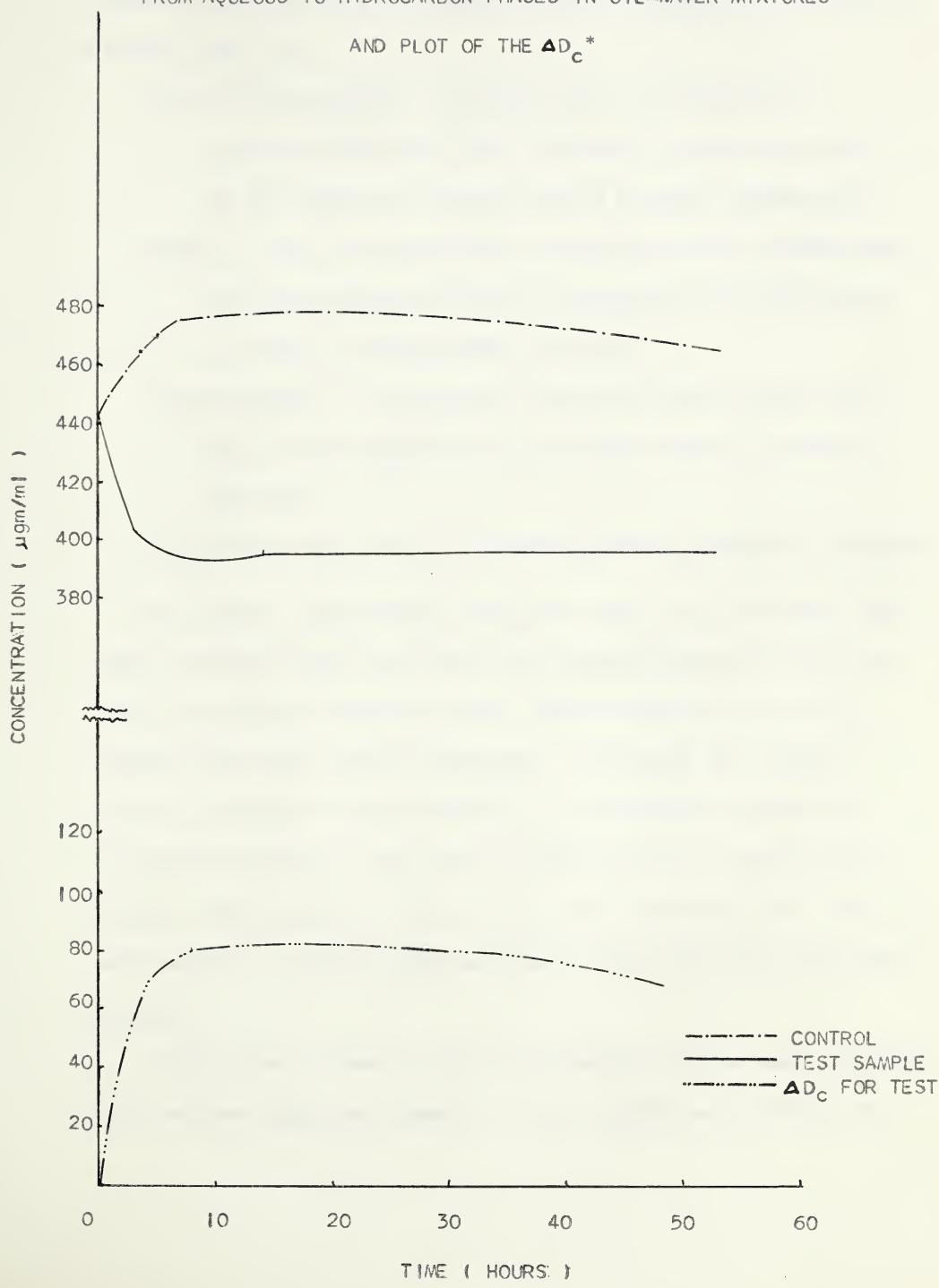
THE EFFECT OF TIME ON THE MIGRATION OF PARA-CHLORO-META-CRESOL FROM
AQUEOUS TO HYDROCARBON PHASES IN OIL-WATER MIXTURES



GRAPH XVIII

THE EFFECT OF TIME ON THE MIGRATION OF PARA-CHLORO-NETA-CRESOL
FROM AQUEOUS TO HYDROCARBON PHASES IN OIL-WATER MIXTURES

AND PLOT OF THE ΔD_c^*



Discussion

In this series of tests maximum migration was considered to have occurred when the ΔD_c became relatively constant. The length of time required for maximum migration varied from system to system. Thus with

2,4,6-trichlorophenol - virtually all of the migration occurred within the first 14 hours of contact and most of the migration occurred within 8 hours, whereas with Phenol - very little migration occurred with this disinfectant but what migration there was happened in the first hour of contact between phases and with

6-chlorothymol - all migration occurred within 8 hours with most of the migration in the first 4 hours of contact and with

Para-chloro-meta-cresol - maximum migration occurred in 8 hours.

The control disinfectant solutions never gave a straight line graph. This may have been due to an oxidation (photo) of the very weak, disinfectant solutions used. Photo-oxidations are to be expected with many phenolic compounds. A scan of the control solutions indicated the occurrence of two absorption peaks for 2,4,6-trichlorophenol, the first at 285 $\text{m}\mu$ and the second peak at 293 $\text{m}\mu$. These anomalies may also have been caused by carry over of small amounts of light liquid petrolatum into the spectrophotometer cuvette.

Slight fluctuations in the concentrations of the test solutions after maximum migration appears to have occurred may indicate the

dynamic state of the disinfectant between the two phases of the system. This may also be caused by slight variations in handling of individual samples.

Correlation Between the Migration of Disinfectant

From An Aqueous Solution and the Loss of Disinfecting

Ability of a Particular System

This test was performed on only one disinfectant, 6-chlorothymol, since about 92% of this disinfectant was shown to migrate from an aqueous to hydrocarbon phase in a 10% hydrocarbon stationary mixture. It was felt that other disinfectants which exhibited partitioning effects would give relatively the same information.

Three systems were prepared for each of the organisms tested. The first system was composed of disinfectant solution, hydrocarbon and test organism. In this mixture, migration of the disinfectant could occur into the hydrocarbon phase thus allowing the organisms to escape the lethal effects of the disinfectant. The second system was a control composed only of disinfectant solution and test organism. This system served as a base line for an unimpeded disinfection study. The third system, also a control, would determine whether the presence of light liquid petrolatum itself had a deleterious effect on the test organisms.

Materials and Method

The test was set up according to the following protocol:
(See TABLE XXIII)

TABLE XXIII

Test Systems Used in Correlating
Disinfectant Migration With Loss of
Disinfecting Ability of a Particular System

Components	Test Mixture	Disinfectant Control(a)	Hydrocarbon Control(b)
Test Organism	1 ml	1.11 ml	1 ml
Disinfectant (Aqueous)	8 ml	8.88 ml	0 ml
Light Liquid Petrolatum	1 ml	0 ml	1 ml
Sterile Water	0 ml	0 ml	8 ml
Total Volume	10 ml	9.99 ml	10 ml

a) Control to study the actual disinfecting ability
of the solution without the influence of hydrocarbon.
b) Control which checks the effect of hydrocarbon on the
test organisms.

Twenty-four hour T.C.S. broth cultures of the test organisms were harvested by centrifugation, suspended in sterile water and a viable count was done using the plate count method. This suspension was used as the inoculum for the test.

After the test organism was placed into the system and before the hydrocarbon was added the system was shaken so as to get a uniform distribution of the constituents. Hydrocarbon was the last component added to the system.

The disinfectant solution contained 200 $\mu\text{gm}/\text{ml}$ of 6-chlorothymol.

At various time intervals, 0.1 ml of the aqueous phase was removed, serially diluted and counted on T.C.S. agar plates at 37°C. Incubation time was 24 - 36 hours.

Staphylococcus aureus F.D.A. 209 and Pseudomonas fluorescens O.A.C. 99 were used as the test organisms. The titre of the bacterial inoculum was:

- a) Staphylococcus aureus - 2.5×10^8 cfu/ml
- b) Pseudomonas fluorescens - 1.75×10^9 cfu/ml

The following two tables serve to indicate the effect of 6-chlorothymol on the test systems being studied.

TABLE XXIV

The Effect of 6-chlorothymol on
Staphylococcus aureus F.D.A. 209

Time (Hours)	System: Dilution:	Test			Control(a)			Control(b)		
		10^{-4}	10^{-5}	10^{-6}	10^{-4}	10^{-5}	10^{-6}	10^{-4}	10^{-5}	10^{-6}
1		1	2	1	1	2	1	TNTC	204	27
2		0	2	0	0	0	0	TNTC	198	35
4		1	0.5	0	0.5	0	0	TNTC	162	18.5

TABLE XXV

The Effect of 6-chlorothymol on
Pseudomonas fluorescens O.A.C. 99

Time (Hours)	System: Dilution:	Test			Control(a)			Control(b)	
		10^{-4}	10^{-5}	10^{-6}	10^{-4}	10^{-5}	10^{-6}	10^{-5}	10^{-6}
1		TNTC	TNTC	95	TNTC	TNTC	110	TNTC	175
2		TNTC	TNTC	39	TNTC	TNTC	40	TNTC	172
4		TNTC	TNTC	45	TNTC	TNTC	50	TNTC	180

Results expressed in colony forming units per ml (cfu/ml)

Results are the average of 2 readings

TNTC - too numerous to count (over 300 cfu/plate)

Discussion

In tests which involved Staphylococcus aureus, no statement can be made concerning the relationship between migration of disinfectant and killing time since the concentration of disinfectant was so great that the organisms were killed before sufficient time had elapsed for significant migration.

With Pseudomonas fluorescens the opposite was found, that is, although the concentration of disinfectant used was sufficient to kill part of the bacterial population in 1 hour even after 4 hours no significant differences between viable counts of the control and the test mixtures were observed.

Control "a", which was the disinfectant control, showed that 200 $\mu\text{gm}/\text{ml}$ of 6-chlorothymol completely inhibits Staphylococcus aureus in about 1 hour and decreases the Pseudomonas population by about 55% in 4 hours.

Control "b", which was the hydrocarbon control, indicated that the presence of hydrocarbon had some deleterious effects on the Staphylococcus since the population was reduced about 34% after 1 hour exposure to the hydrocarbon. The Pseudomonas population was unaffected by the hydrocarbon presence.

Since we were unable to show any effect of migration of disinfectant on killing time by adding the test organisms to the mixture of hydrocarbon and aqueous disinfectant solution at the time of mixing we decided to modify the experiment by mixing the hydrocarbon and aqueous disinfectant solution prior to the addition of the test organisms thus allowing time for possible migration to occur.

To test the validity of our decision to modify the experiment as indicated above, two solutions were prepared. Test system I contained aqueous disinfectant solution and hydrocarbon in which migration was allowed to occur from the aqueous phase to the hydrocarbon phase before the test organisms were added. The killing time of this solution was compared to the killing time of an aqueous phase which was placed in contact with light liquid petrolatum at the same time as the test organisms were added.

The contact time for the two phases of solution I, was 8 hours.

The test systems were set up according to the following protocol:

TABLE XXVI

Test Systems Required in Correlating
Disinfectant Migration With Loss of Disinfecting
Ability of Aqueous Disinfectant Solutions

<u>Components</u>	<u>System I*</u> (mls.)	<u>System II</u> (mls.)	<u>Control</u> (mls.)
Light Liquid Petrolatum	0	1	1
Aqueous Disinfectant	8	8	0
<u>Staphylococcus</u> Suspension	1	1	1
Sterile Water	1	0	8
Total Volume	10	10	10

* The 8 ml of disinfectant solution used in System I had been in contact with 10% hydrocarbon for 8 hours prior to the experiments.

- The test organisms were the last component added to each system.

Using 6-chlorothymol (200 $\mu\text{gm}/\text{ml}$), para-chloro-meta-cresol (450 $\mu\text{gm}/\text{ml}$) and 2,4,6-trichlorophenol (200 $\mu\text{gm}/\text{ml}$) as the disinfectants, Staphylococcus aureus was employed as the test organism. Pseudomonas fluorescens O.A.C. 99 was used as the test organism only when para-chloro-meta-cresol was the disinfectant being tested since the concentration of the other disinfectants was too low to inhibit Pseudomonas.

At intervals of 0, 1, 2 and 4 hours for 6-chlorothymol and 6, 10, 12 and 24 hours for the other 3 disinfectants, 0.1 ml of the aqueous phase was removed from each test system (I and II) and control, serially diluted and plate counted on T.C.S. agar. Incubation was for 24 hours at 37°C.

Results

The results of these experiments are given in TABLE XXVII.

TABLE XXVII

A Comparison of the Disinfecting Ability
of Two Solutions of Aqueous Disinfectant
Which Have Been Treated Differently

Time (hours)	System: Dilution:	System I			System II			Control		
		10 ⁻³	10 ⁻⁴	10 ⁻⁵	10 ⁻³	10 ⁻⁴	10 ⁻⁵	10 ⁻⁵	10 ⁻⁶	10 ⁻⁷

6-chlorothymol (200 $\mu\text{gm}/\text{ml}$) Tested on Staphylococcus aureus

1	TNTC	TNTC	393	19	1	0	TNTC	TNTC	164
2	TNTC	TNTC	TNTC	71	4	0	TNTC	TNTC	170
4	TNTC	TNTC	TNTC	127	8	1	TNTC	TNTC	160

P-C1-M-Cresol (450 $\mu\text{gm}/\text{ml}$) Tested on Staphylococcus aureus

6	50	5.5	1	25	2	0	224
10	0	2	0	0	0	0	230
12	1	0	0	0	0	0	250
24	0	0	0	0	0	0	228

TABLE XXVII

A Comparison of the Disinfecting Ability
of Two Solutions of Aqueous Disinfectant
Which have been Treated Differently

Time (hours)	System: Dilution:	System I			System II			Control		
		10 ⁻³	10 ⁻⁴	10 ⁻⁵	10 ⁻³	10 ⁻⁴	10 ⁻⁵	10 ⁻⁵	10 ⁻⁶	10 ⁻⁷

Cont.

P-C1-M-Cresol (450 µgm/ml) Tested on Pseudomonas fluorescens

6	TNTC	97	15	-	33.5	4	281
10	TNTC	53	6	-	22	2	260
12	TNTC	62.5	5	300	30	3.5	255
24	TNTC	58.5	-	319	40	-	250

2,4,6-trichlorophenol (200 µgm/ml) Tested on Staphylococcus aureus

6	TNTC	TNTC	157	30	4	0	224
10	TNTC	TNTC	247	0	0	0	230
12	TNTC	TNTC	272	0	0	0	230
24	TNTC	TNTC	277.5	0	0	0	250

- = no reading taken at this dilution

0 = no viable cells at this dilution

I = 6-chlorothymol had 8 hours prior exposure to hydrocarbon
and the other disinfectants had 12 hours of prior
exposure.

II = In System II the organisms and hydrocarbon were added
to the disinfectant solution at the same time.

Results expressed in cfu/ml (colony forming units)

Discussion of Results

6-chlorothymol

The bactericidal ability of the 6-chlorothymol solution was markedly reduced by the 8 hour exposure of the disinfectant solution to the light liquid petrolatum (System I). This loss of ability of the disinfectant to kill the test organisms, is related to the migration of the disinfectant into the hydrocarbon phase which was

previously demonstrated spectrophotometrically.

By comparing the titre of the control mixture with Test System I, it may be seen that the organisms are affected slightly by the small amount of disinfectant present but after a short time multiplication is virtually uninhibited. System II, which did not have prior exposure to hydrocarbon inhibited Staphylococcus greatly in the first hour of exposure but multiplication ensued quickly indicating that migration of the disinfectant was occurring.

Since a concentration of 200 $\mu\text{gm}/\text{ml}$ of 6-chlorothymol is nearing the saturation point in water at room temperature, it is obvious that this disinfectant would not be suitable for disinfecting a preparation or system which contained a hydrocarbon phase. Although the mixture might be disinfected initially, as time proceeded the 6-chlorothymol would migrate from the aqueous phase leaving the water bottom deficient in disinfectant and thus suitable for microbial survival.

Para-chloro-meta-cresol

When the disinfectant solution was in contact with 10% light liquid petrolatum for 12 hours prior to the test, some migration occurred. This has been shown in previous spectrophotometric experiments where it was seen that maximum migration of para-chloro-meta-cresol from an aqueous solution into 10% hydrocarbon occurred in 10 hours. (See GRAPH XIV). Staphylococcus aureus was able to survive in the pretreated system (System I) for at least 12 hours whereas in the second (System II) system survival was not evident at 10 hours.

Where Pseudomonas fluorescens was used as the test organism, it survived at least 24 hours in both systems.

There was no significant difference between the viable counts in System I and System II when either Staphylococcus aureus or Pseudomonas fluorescens was used as the test organism.

2,4,6-trichlorophenol

The results obtained using Staphylococcus aureus are very convincing as to the effect of disinfectant migration on microbial survival. In systems which had been pretreated with light liquid petrolatum (System I), no significant alteration in the bacterial population occurred in 24 hours. In sharp contrast to this, the untreated system (System II) had a count of only 3.0×10^5 organisms per ml after 6 hours and less than 1×10^3 organisms per ml after 10, 12 and 24 hours.

Conclusion

It is very evident from experiments using aqueous disinfectant solutions which had been in contact with light liquid petrolatum before the bacteria were added and those which were placed in contact with the hydrocarbon at the same time as the bacterial inoculum was added, that migration of a disinfectant from an aqueous phase to a hydrocarbon phase is an important factor in determining the efficiency of disinfection.

Our experiments involving 6-chlorothymol, and 2,4,6-trichlorophenol support this claim whereas experiments with para-chloro-meta-cresol do not give supporting evidence for the claim that migration of a disinfectant from an aqueous phase to a hydrocarbon phase is an important factor in disinfection of oil-water mixtures.

The Effect of Temperature on the Migration of
Disinfectants From an Aqueous Phase to
a Hydrocarbon Phase

One of the prime factors affecting solubility and partitioning functions of a chemical is temperature.

To study the rate of migration of disinfectants as a function of temperature, aqueous solutions of disinfectant were placed in contact with light liquid petrolatum. These mixtures were incubated at various temperatures and spectrophotometric readings taken at various time intervals.

The following table and outline describe the procedure we used in studying the effect of temperature on the migration of disinfectants from an aqueous phase to a hydrocarbon phase of a hydrocarbon-water mixture.

TABLE XXVIII

Properties of the Disinfectants Used in Studying
 The Effect of Temperature on Partitioning

<u>Disinfectant</u>	<u>Concentration</u>	<u>Absorption Maximum</u>
2,4,6-trichloropheno1	200 μ gm/ml	285 μ
6-chlorothymol	200 μ gm/ml	281 μ
Para-chloro-meta-cresol	450 μ gm/ml	226 μ

One ml of the hydrocarbon was layered on 9 ml of the aqueous disinfectant solution in a 50 ml Erlenmeyer flask. One set of flasks, containing the above mentioned mixture, were maintained at 37°C, one set at 6°C and another set were maintained at room temperature (26°C) or 30°C as in the case of para-chloro-meta-cresol. A sufficient number of replicate mixtures were prepared for each

disinfectant so that at various time intervals spectrophotometric readings could be made of the aqueous phase of one of the replicate mixtures of each disinfectant which had been held at each of the three temperatures without disturbing the remaining mixtures.

The results of these experiments are given in TABLES XXIX, XXX, XXXI and illustrated graphically in GRAPHS XIX, XX and XXI. The results reported in both the graphs and the table are in optical density units. In order to obtain actual concentrations the optical density values may be compared to the standard curves of optical density versus concentration.

TABLE XXIX

The Effect of Temperature on the
Migration of 6-chlorothymol

Time (Hours)	Optical Density of Aqueous Phase at:			
	37°C	26°C	6°C	Control*
0	-	-	-	1.85
2	0.20	0.65	0.75	1.80
4	0.14	0.18	0.615	1.85
8	0.12	0.165	0.505	1.85
24	0.095	0.095	0.20	1.90
52	0.095	0.11	0.145	1.85

* the control contained no hydrocarbon

TABLE XXX

The Effect of Temperature on the
Migration of 2,4,6-trichlorophenol

Time (Hours)	Optical Density of the Aqueous Phase at:			
	37°C	26°C	6°C	Control*
0	-	-	-	1.50
2	0.275	0.475	0.55	1.465
4	0.16	0.175	0.51	1.45
8	0.11	0.115	0.27	1.38
24	0.125	0.130	0.185	1.315
52	0.17	0.165	0.20	1.23

* Control contained no hydrocarbon

TABLE XXXI

The Effect of Temperature on the
Migration of Para-chloro-meta-cresol

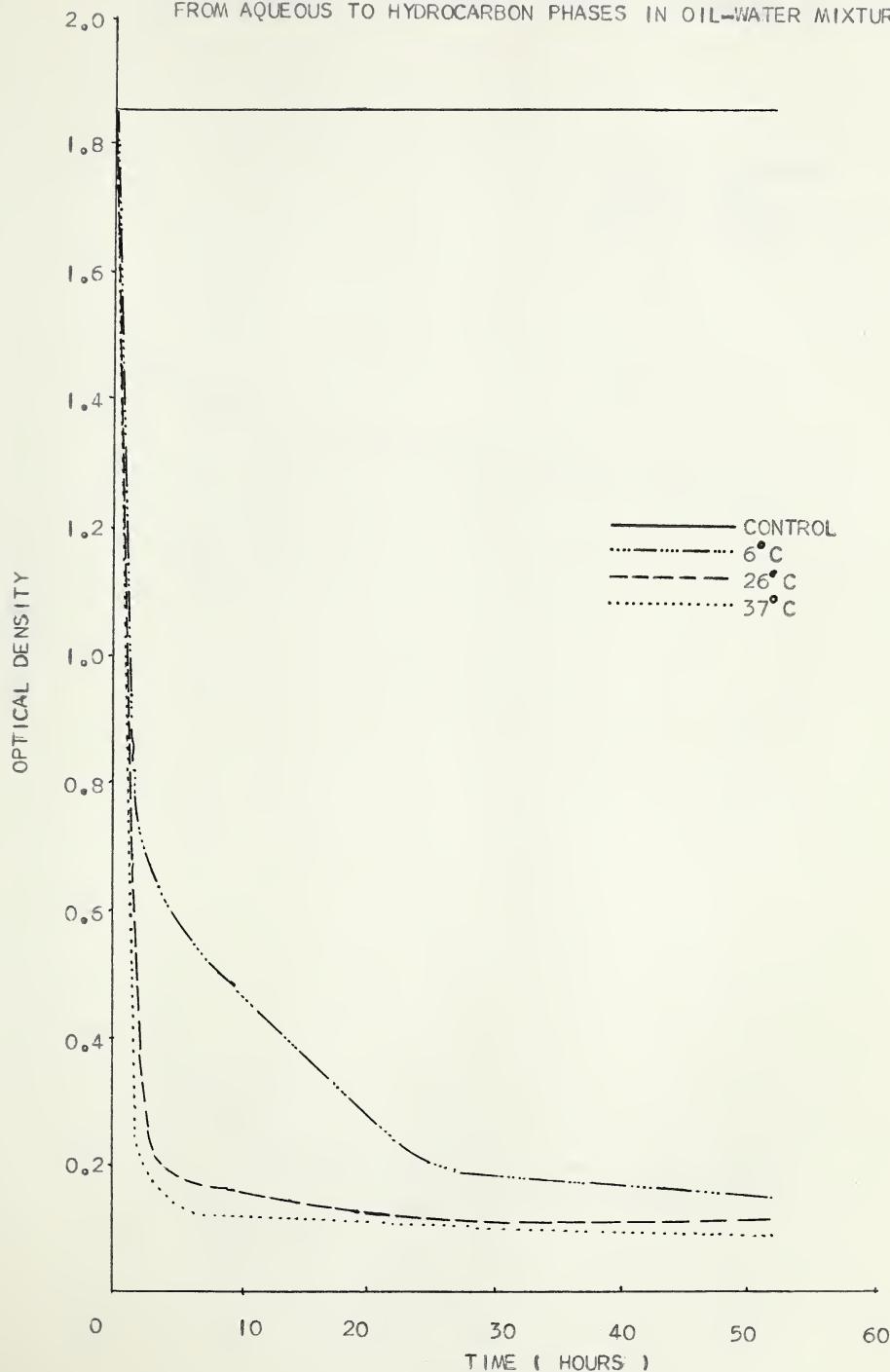
Time (Hours)	Optical Density of the Aqueous Phase at:			
	37°C	30°C	6°C	Control*
1.5	0.425	0.445	0.475	0.495
3.5	0.41	0.415	0.46	0.495
5.0	0.42	0.42	0.44	0.49
6.0	0.40	0.405	0.44	-
8.0	0.40	0.415	0.46	0.50
27.0	0.44	0.415	0.45	0.50

* Control contained no hydrocarbon

GRAPH XIX

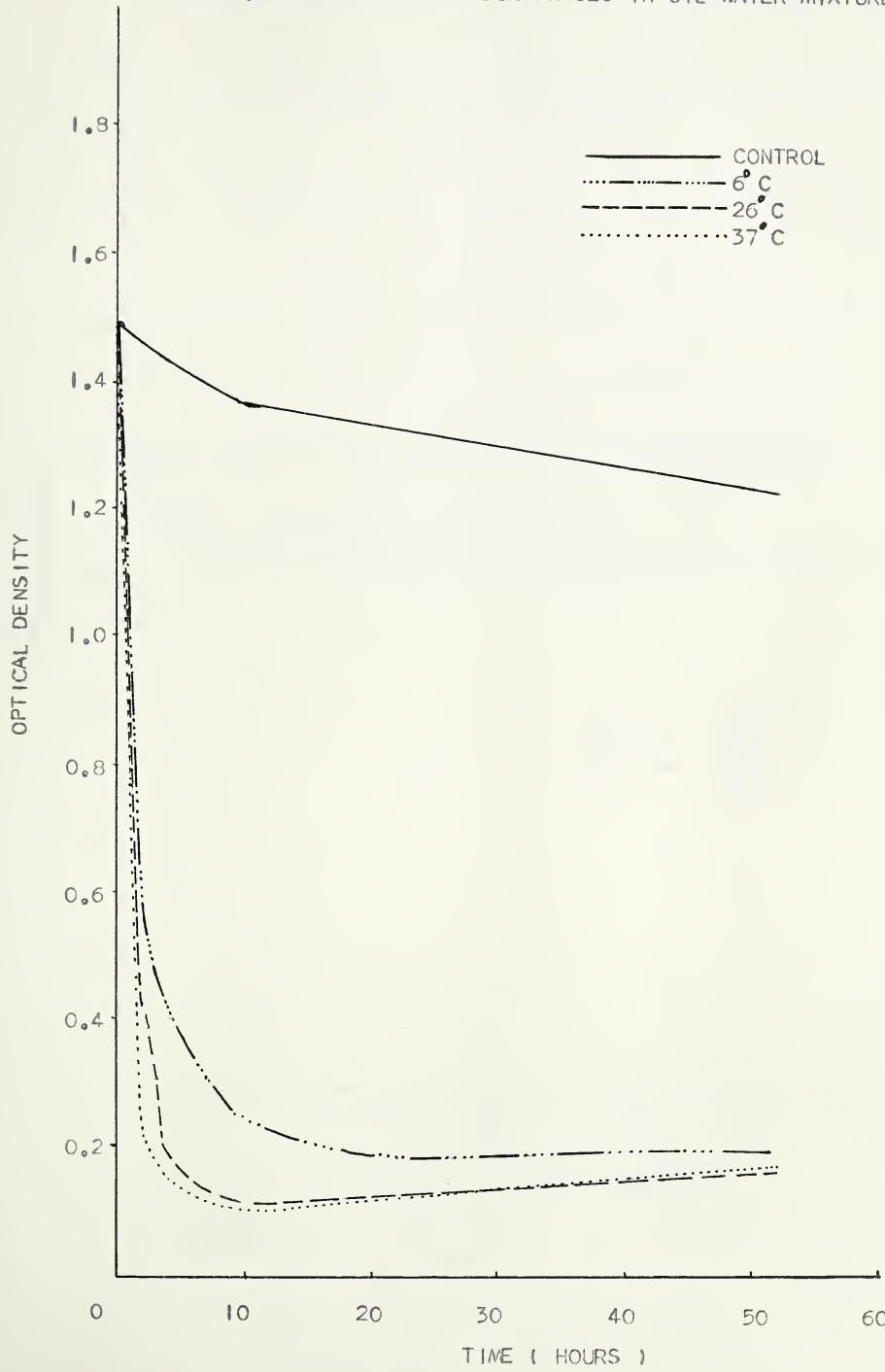
THE EFFECT OF TEMPERATURE ON THE MIGRATION OF 6-CHLOROTHYMPH

FROM AQUEOUS TO HYDROCARBON PHASES IN OIL-WATER MIXTURES



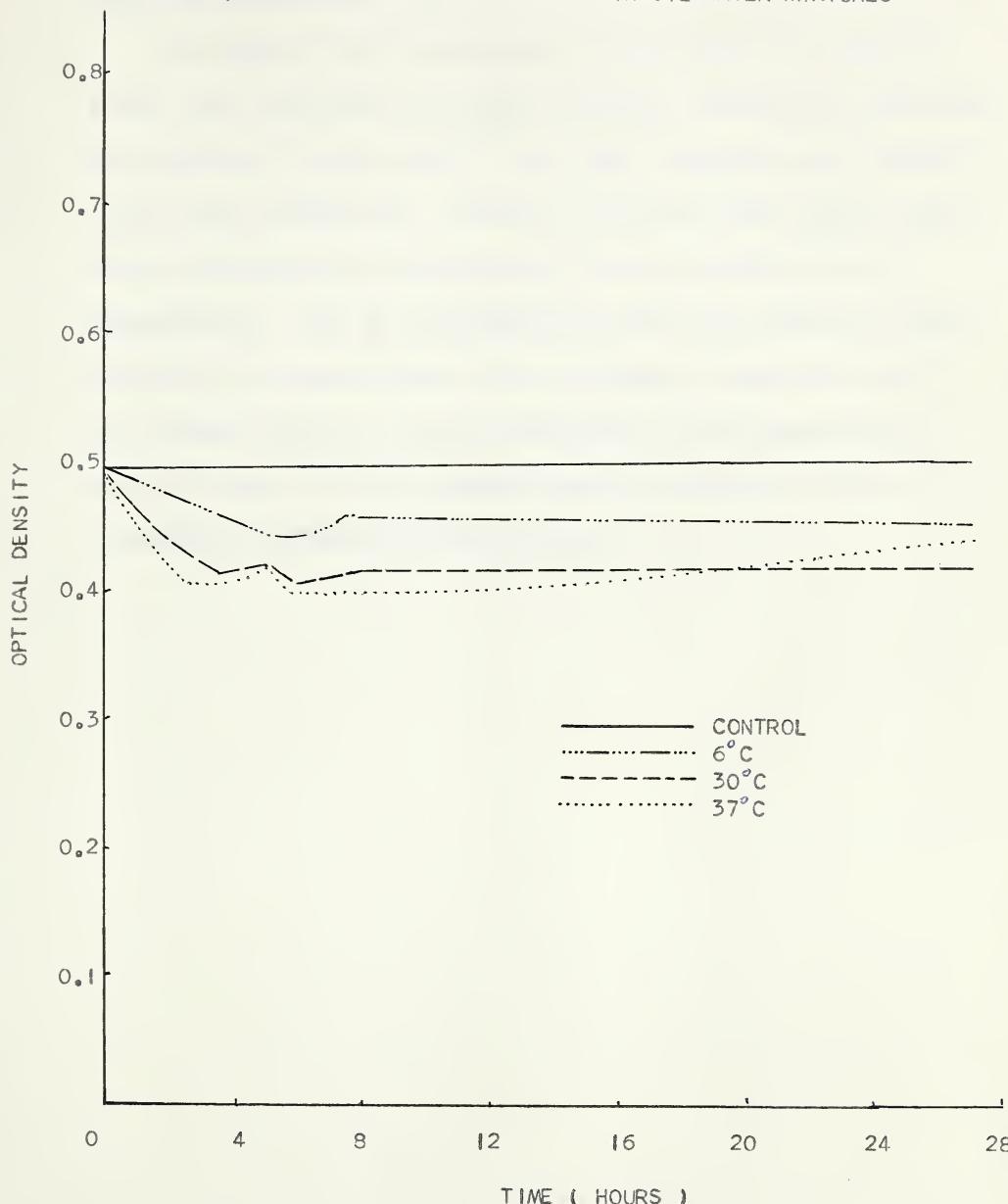
GRAPH XX

THE EFFECT OF TEMPERATURE ON THE MIGRATION OF 2,4,6-TRICHLOROPHENOL
FROM AQUEOUS TO HYDROCARBON PHASES IN OIL-WATER MIXTURES



GRAPH XXI

THE EFFECT OF TEMPERATURE ON THE MIGRATION OF PARA-CHLORO-META-CRESOL
FROM AQUEOUS TO HYDROCARBON PHASES IN OIL-WATER MIXTURES



Discussion

This data indicates that the rate of migration of disinfectants from aqueous solutions into hydrocarbon phases is influenced by varying temperatures.

All three of the disinfectants studied behave in a similar manner when subjected to the three different temperature conditions. The migration is most rapid at the higher temperature and slowest at the lower temperature. However, the final result (that is the final concentration of disinfectant) is very similar for all temperatures. This is to be expected since both phases are being affected in the same manner thus any change in temperature will only change the rate of partitioning and not the quantitative picture unless one of the phases reaches a saturation point at a particular temperature being studied.

CHAPTER III

A Study of the Lipid and Amino Acid Composition
of Pseudomonas fluorescens Grown in
Light Liquid Petrolatum-Mineral Salts
Medium and Trypticase Soy Broth

The Lipids and Amino Acids of
Pseudomonas fluorescens O.A.C. 99

Much of the work of this thesis involved Pseudomonas fluorescens grown in light liquid petrolatum - mineral salts medium. The growth of this organism was very slow when compared to its growth in more complex media such as trypticase soy broth. Only by using long incubation times, constant agitation and aeration was it possible to get high enough titres of Pseudomonas for most of the studies we performed. Because of the low titres obtained when grown in light liquid petrolatum - mineral salts medium it became obvious that this Pseudomonas could use light liquid petrolatum as a carbon and energy source only with great difficulty. We therefore decided to investigate the differences in amino-acid pools and lipid composition of this organism when grown in a simple and a complex medium, since these differences may give some explanation for differences in the growth response.

LIPIDS

Introduction

The lipid composition of microorganisms varies greatly both in composition and relative quantities of a particular component between groups of organisms. However, bacteria do not generally synthesize polyunsaturated fatty acids and, therefore contain mainly monounsaturated and saturated fatty acids that are in many cases branched or contain ring structures (Korn, 1964).

Besides free fatty acids, the microbial cell can contain phospholipids, mono, di and triglycerides, glycolipids, peptidolipids and derivatives thereof. (Kaneshiro and Marr, 1962). Thus the possible number of lipid components in a single cell can be vast and complicated. Korn (1964) has determined the complete structure of 51 fatty acids alone from Euglena gracilis. Lederer (1964) has done extensive study on the lipids of Mycobacterium tuberculosis.

There are many methods cited in the literature for the separation of lipid components. Included are such methods as fractional distillation, spectrophotometry, chromatography (paper, column, thin-layer, gas-liquid) and countercurrent distribution (Coleman, 1963). These methods all suffer in their inability to resolve completely, very complex mixtures. (when used individually)

We wished to combine the use of thin-layer adsorption chromatography, for the fractionation of groups of lipids, as a method complementary to gas-liquid chromatography. This procedure has been used by Mangold and Kammerbeck (1961) in the separation, identification and quantitative analysis of fatty acid mixtures.

Identification of Lipid Components of *Pseudomonas fluorescens*

O.A.C. 99 Grown in Trypticase Soy Broth

Culture

Pseudomonas fluorescens O.A.C. 99 was cultured in T.C.S. broth at 37°C under aerobic conditions. The culture was incubated for 72 hours.

Extraction of Lipid

The bacterial cells were recovered from the culture medium by centrifugation in the cold (6°C) and washed with sterile water twice to remove residual culture material. The cells were

then air-dried at room temperature in vacuo, weighed and ball-mill extracted using a Vir-Tis "23" homogenizer operated at 14,000 r.p.m. with glass beads (see Lamanna and Mallette, 1954).

The cells were extracted twice for 2 hours at room temperature (22 - 25°C) with each solvent system according to the following extraction scheme (TABLE XXXII)

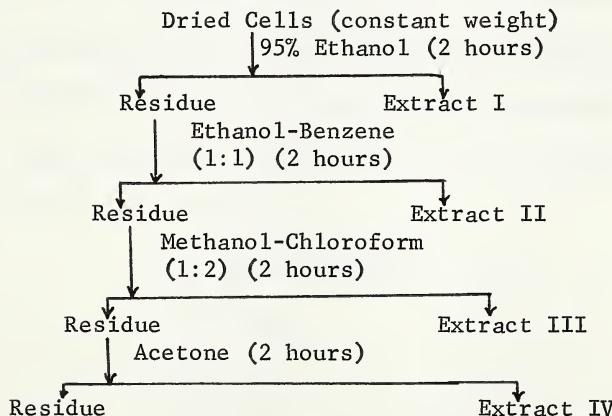
The following solvent systems were employed:

- 1) 95% Ethanol (extracts most protein bound lipid)
- 2) Ethanol Benzene (1:1^{v/v})
- 3) Methanol-Chloroform (1:2^{v/v})
- 4) Acetone

The duplicate extracts were pooled and solvents removed by air-drying in vacuo at room temperature.

TABLE XXXII

Lipid Extraction Scheme for
Pseudomonas fluorescens O.A.C. 99



After evaporating the solvent from each of the four extracts, the containers were weighed. The difference in weight of the flask before the extracted material was added and the weight after evaporation of solvent represented lipids.

The lipid was then dissolved in chloroform and the four extractions pooled. The pooled chloroform solution was washed twice with 1N hydrochloric acid to remove any basic materials. The washed chloroform solution of lipid was then re-evaporated and weighed. This chloroform-soluble fraction represented the total extractable lipid.

Thin-Layer Chromatography

Preparation of Thin-Layer Plates

It was decided to use neutral thin-layer plates instead of acid plates. Therefore 0.1N sodium carbonate (Na_2CO_3) was used instead of water in the preparation of the plates. The sodium carbonate neutralizes salicic acid. The addition of 1,2 dimethoxyethane allows the preparation of thicker plates and these in turn allow for better resolution of components (preparative plates). The ingredients required for 2 neutral plates 1 mm thick \times 20 cm \times 20 cm are as follows:

1,2 dimethoxyethane 20 ml

0.1N Na_2CO_3 80 ml

Silica gel 50 gm

The silica gel is added to the two liquids and triturated quickly into a uniform smooth paste. The paste is then spread evenly on the glass plates and allowed to set at room temperature. The plates are then charged at 100°C for 4 hours in a hot-air drying oven.

Solvent Systems Employed in Thin-Layer Chromatography

The following solvent systems were employed in our thin-layer chromatography of the lipids of Pseudomonas fluorescens.

Solvent System I (Mangold and Kammerer, 1961)

n-hexane 4

Diethyl ether 1

This system was allowed to ascend for 1.5 hours covering a distance of 16 cm.

Solvent System II (Mangold and Kammerer, 1961)

Glacial Acetic Acid 1

n-propanol 100

Allowed to ascend for 4.5 hours.

Solvent System III

Chloroform 60

Methanol 35

7N Ammonium hydroxide 6

Detection Reagents Employed in Thin-Layer Chromatography

The detection reagents employed in the localization of lipids in our thin-layer chromatography work were:

Iodine Vapors

The thin-layer plates are allowed to sit in a covered vessel with iodine crystals for a few minutes. Lipid materials show up as light brown bands. If the plates are allowed to sit in the air for a short time, the iodine sublimes off the plate.

Ninhydrin Solution

Ninhydrin 0.2 gm

Acetone 100 ml

A 0.2% ninhydrin solution can be used to detect free amino compounds by spraying on the plates and heating for 5 minutes at 100°C in a hot-air drying oven.

Sodium Fluorescein Solution

Sodium fluorescein 0.4 gm

Distilled water 100 ml

Free fatty acids and triglycerides show up as reddish-pink spots under ordinary light but as brown spots under ultra-violet light. Triethanolamine is whitish-green under ordinary light.

Preparation of Standards and Evaluation of Solvent Systems

A number of straight chain saturated fatty acids and triglycerides were prepared to be used as standards for the evaluation of the three solvent systems chosen for this study.

"A" Standards for Saturated Straight Chain Fatty Acids

The following fatty acids were dissolved in chloroform:

- 1) Caproic acid - C8
- 2) Capric acid - C10
- 3) Lauric acid - C12
- 4) Palmitic acid - C16
- 5) Stearic acid - C18

"B" Standards for Triglycerides

The following triglycerides were dissolved in chloroform:

- 1) Trilaurin
- 2) Trimyristin
- 3) Tripalmitin
- 4) Triethanolamine

A chloroform solution of the "A" standards was applied along one side of the plate, 2 cm from the edge and a chloroform solution of the "B" standards was applied along one edge of a second plate. The samples were placed about 2 cm from the base of the plate to allow a free area for immersion into the solvent system.

The plates were first subjected to the n-hexane-diethyl-ether solvent for 1.5 hours then dried at room temperature and exposed to iodine vapors. After the results were recorded and the iodine had sublimed off the plates, the plates were run again in the same dimension, in n-propanol-glacial acetic acid for 4 hours. The bands were again developed with iodine vapors.

The next experiment involved preparing chloroform solutions of 9 different straight chain fatty acids, triglycerides and triethanolamine. These solutions were applied on thin-layer plates as spots and developed for 2 hours in chloroform-methanol-ammonium hydroxide at room temperature. The plates were removed from the chromatography tank and allowed to dry at room temperature. The spots were detected using a spray of 0.4% sodium fluorescein. The results of experiments with the different solvent systems and different fatty acid mixtures are given in

TABLE XXXIII.

TABLE XXXIII

Evaluation of Two Solvent Systems for the Separation of a Mixture of Fatty Acids

<u>Standard "A"</u>	<u>Solvent Systems</u>	<u>Character of Chromatogram</u>
Caproic acid	n-hexane-diethyl ether	1 band 1.5 cm from the origin after 1½ hours. Solvent front 16.2 cm from the origin.
Capric acid		
Lauric acid		
Palmitic acid		
Stearic acid		
	n-propanol-glacial acetic acid	After 4 hours there were 4 bands at 9.2 cm, 10.2 cm, 11.0 cm, and 13.5 cm. Solvent front 16 cm.
<u>Standard "B"</u>		
Trilaurin	n-hexane-diethyl ether	1 band 0.4 cm from origin after 1½ hours. Solvent front 16.2 cm from the origin.
Trimyristin		
Tripalmitin		
Triethanolamine		
	n-propanol-glacial acetic acid	After 4 hours there were 3 bands at 11 cm, 14.5 cm, 15.1 cm. Solvent front 16 cm.

To investigate the possible reason for the lack of separation when mixtures of fatty acids were used, a third solvent system was used. Nine different fatty acids (the components of Standards A and B) were spotted individually along the base of a thin-layer plate.

The following Table serves to indicate the reason for lack of separation of mixtures of fatty acids in a single solvent system.

TABLE XXXIV

Movement of 9 Different Lipids Using
Chloroform-Methanol-7N Ammonium Hydroxide

<u>Lipid</u>	<u>Cm from Origin*</u>
Capric acid	6.2
Tripalmitin	15.0
Triethanolamine	0.5 - 8.4
Caproic acid	6.7
Stearic acid	5 - 7.5
Lauric acid	6.4
Trilaurin	14.6
Trimyristin	14.9
Palmitic acid	6.0

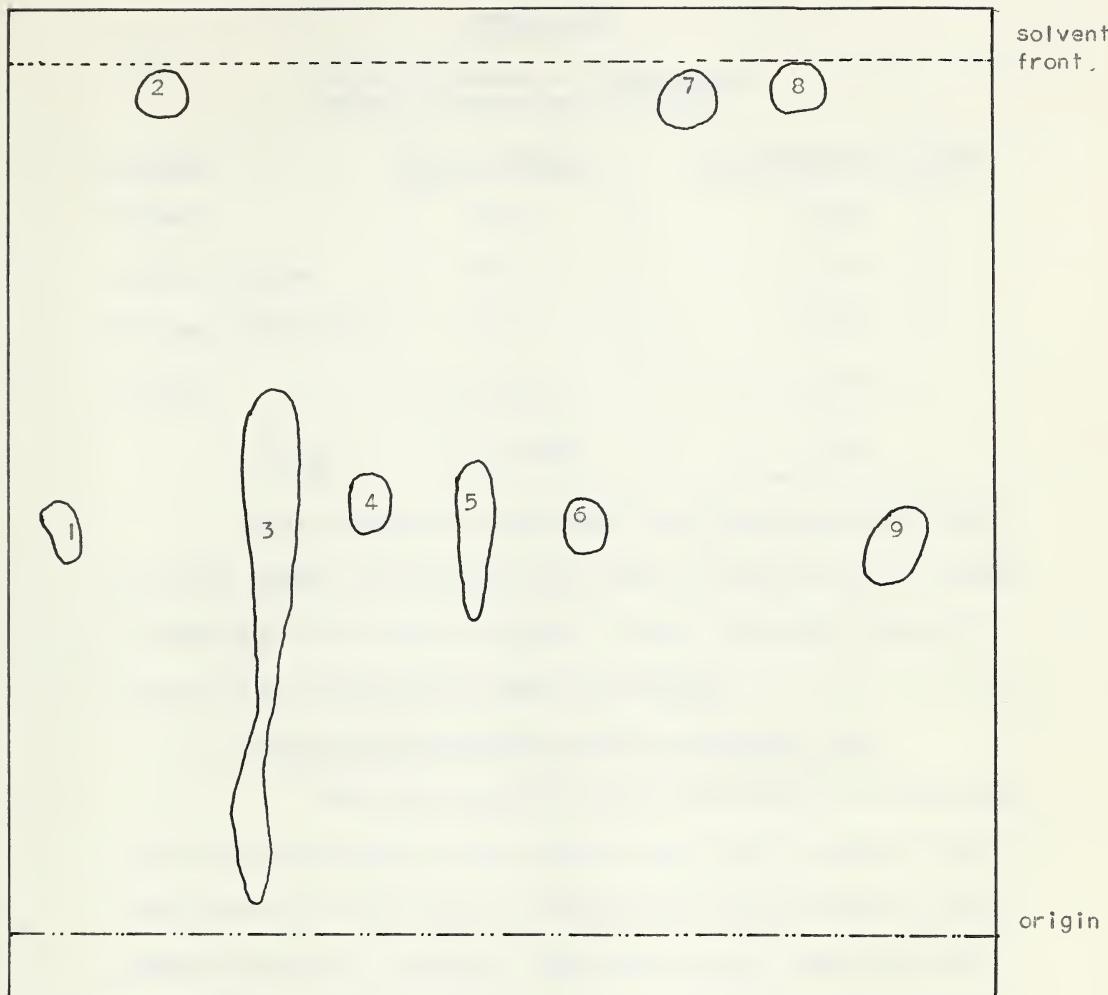
* Solvent front was 15.4 cm from the origin
in 2 hours at room temperature.

Figure II is a tracing of the thin-layer chromatography plate used in studying the movement of 9 different lipids in Solvent System III.

It is very evident from the foregoing experiments that free fatty acids and other lipids in mixtures can not be separated completely into single components by the use of any of the three solvent systems studied. The use of Solvent III ($\text{CHCl}_3\text{-CH}_3\text{OH-7NNH}_4\text{OH}$) however allowed for good separation of free straight chain fatty acids from triglycerides. This separation was quite rapid. On this basis we decided to use Solvent System III for the analysis of the unknown lipid extracted from Pseudomonas fluorescens O.A.C. 99.

FIGURE II

THIN-LAYER CHROMATOGRAM OF 9 LIPIDS



Chromatogram of straight chain saturated fatty acids and triglycerides in the chloroform-methanol-ammonium hydroxide solvent. (1) Caprylic acid, (2) Tripalmitin, (3) Triethanolamine, (4) Caproic acid, (5) Stearic acid, (6) Lauric acid, (7) Tritaurin, (8) Trimyristin, (9) Palmitic acid. Fatty acids were detected with iodine vapors.

Results of Lipid Extraction Procedure

The amount of lipid extracted from 0.5871 gm (dry weight) of Pseudomonas cells is given in TABLE XXXV.

TABLE XXXV

Lipid Extraction of Pseudomonas
Grown in Trypticase Soy Broth

<u>Solvent</u>	<u>Mgm of Extract</u>	<u>% Dry Weight of Cells</u>
Ethanol	32.8	5.58
Ethanol-benzene	25.5	4.34
Methanol-chloroform	12.1	2.06
Acetone	0.4	0.07
Total	70.8 Mgm	12.05%

After washing the combined lipid fractions with 1N HCl, the total weight of lipid was 68.7 mgm or 11.70% of the dry weight (0.5871 gm) of the cells extracted. Some of the acid soluble material was thought to be bacterial pigment.

Thin-Layer Characterization of Extracted Lipid

The 68.7 mgm of lipid was dissolved in a very small amount of chloroform and then spotted onto T.L.C. plates. The plates were run for 2 hours in Solvent III and developed in 0.4% sodium fluorescein yielding 9 bands each on two plates as shown in TABLE XXXVI.

TABLE XXXVI

Separation of a Lipid Fraction of Pseudomonas fluorescens O.A.C. 99 Using Thin-Layer Chromatography

Band	Plate I*		Plate II**	
		Cm From Origin		Cm from Origin
1		0.8	1	1.2
2		1.3	2	2.0
3		4.0	3	4.5
4		5.3	4	6.4
5		7.5	5	8.3
6		8.2	6	9.5
7		10.7	7	12.4
8		13.0	8	14.6
9		14.2	9	15.3

* Solvent front 16 cm

** Solvent front 16.9 cm

Under short ultra-violet light (Mineralight Ultra-Violet Lamp Short Wave SL-2537), band 7 is blue whereas the other bands are brownish-black. Some bands are much darker than others.

Analysis by T.L.C. of the 1N HCl Fraction Used to Remove Basic Materials From the Lipid Extract.

The aqueous solution was spotted directly onto the T.L.C. plate and developed, 2 hours in chloroform-methanol-ammonium hydroxide. The plates were stained with iodine vapors and 0.2% ninhydrin solution. Two bands developed when stained with iodine (15.5 and 14.5 cm from origin with a solvent front at 15.6 cm) and two bands developed when sprayed with ninhydrin (one band at 1 cm and one at 3.5 cm with a solvent front at 15.6 cm).

The band at 14.5 cm showed a distinct green color even without staining and was probably the Pseudomonas pigment pyocyanine. The ninhydrin positive bands indicated acid soluble compounds containing free amino groups.

Discussion

Three solvent systems were studied for the thin-layer chromatographic separation of bacterial lipids. Of these chloroform-methanol-ammonium hydroxide gave the best results.

The 9 bands obtained from the unknown sample and the underlying gel were scraped off the glass plates and extracted with chloroform. These fractions were saved for analysis by gas-liquid chromatography.

Cells of Pseudomonas fluorescens O.A.C. 99 grown in trypticase soy broth under the described conditions, contain 11.70% total extractable lipid.

The Identification of Lipid Components of *Pseudomonas fluorescens*

Grown on Light Liquid Petrolatum - Mineral Salts Medium.

Culturing Technique

The Pseudomonas inoculum was grown at 37°C in the mineral salts - hydrocarbon medium of Raymond and Davis (1960) and contained the following constituents per 1000 ml final volume of medium.

	<u>Grams</u>
$(\text{NH}_4)_2 \text{SO}_4$	1.0
$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	0.2
$\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$	0.005
KH_2PO_4	2.0
Na_2HPO_4	3.0
Na_2CO_3	0.1
CaCl_2	0.01
MnSO_4	0.002
* Yeast Extract	0.02
Light Liquid Petrolatum	8.0

* Raymond and Davis used 0.008 gm of ashed yeast extract.

The salts were autoclaved separate from the water and solution was initiated when both fractions had cooled.

The culture was transferred 3 times at 48 hour intervals before use as inoculum in the final experiment. The repeated transfers should allow time for adaptation of the bacteria from a complex trypticase soy medium to the relatively simple medium shown above.

Following the third transfer of organisms, they were inoculated to 1 litre Erlenmeyer flasks containing 500 ml of sterile mineral salts - hydrocarbon medium. The inoculated flasks were incubated at 37°C with constant aeration and mixing (magnetic - stirring device).

After 5 days incubation the cells were removed from the culture medium by centrifugation and washed twice with distilled water followed by a very superficial washing with n-hexane to remove residual substrate hydrocarbon.

The harvested cells were dried to constant weight in vacuo and ball mill-extracted according to the aforementioned scheme.

Results

From 57.4 mgm dry weight of Pseudomonas cells was extracted 8.3 mgm of lipid. The amount extracted by each solvent system is shown in TABLE XXXVII.

TABLE XXXVII

Lipid Extraction of Pseudomonas Grown in
Mineral Salts - Light Liquid Petrolatum

<u>Solvent</u>	<u>Mgm of Extract</u>	<u>%Dry Weight of Cells</u>
Acetone	7.70	13.41
Ethanol-benzene	0.60	1.05
Methanol-chloroform	0.00	0.00
Ethanol	0.00	0.00
<u>Total</u>	<u>8.30</u>	<u>14.46</u>

The total weight of the material extracted from 57.4 mgm (dry weight) of cells represents 14.46% of the total cells weight.

The extracted materials were next dissolved in chloroform and washed with 1N hydrochloric acid to remove basic materials. The remaining chloroform was evaporated yielding 8.1 mgm of lipid material or 14.11% of the dry weight of the cells.

It was noticed that cells grown on mineral salts - hydrocarbon medium were much less pigmented than those grown in trypticase soy broth. This deficiency of pigment may account for the small amount of 1N HCl soluble materials as compared to the amount in T.C.S. broth grown cells.

We ~~had~~ anticipated the use of a gas - liquid chromatograph for the identification of lipid components extracted from Pseudomonas fluorescens O.A.C. 99, however the apparatus was not available at the time of this investigation, thus the preparative separation performed on thin-layer chromatography yielded no significant information to us.

Discussion

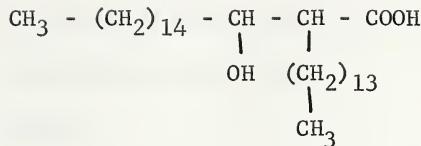
The lipid content of cells grown in trypticase soy broth (T.C.S.) was found to be 11.7% dry weight of the cells. An increase in cellular lipid content was observed when Pseudomonas fluorescens was grown in mineral salts - light liquid petrolatum medium. That is, 11.7% lipid in T.C.S. broth grown cells and 14.11% lipid in the cells grown in hydrocarbon containing medium. This represents an increase of 2.41% extractable lipid.

The Pseudomonas probably synthesized a variety of compounds from the light liquid petrolatum including fats, proteins, nucleic acids, amino acids, and carbohydrates. The most likely point to start obtaining energy and carbon for these syntheses is the terminal carbon of the alkane molecule, followed by β -oxidation of the carbon chain and utilization of the released energy and C_2 fragments for biosynthetic reactions.

Thijsse and Van Der Linden (1958, 1961), using a strain of Pseudomonas aeruginosa, found that primary attack on the alkane molecule is on the α -carbon exclusively. Oxidation of the terminal methyl group yielded the fatty acid which was subsequently cleaved by β -oxidation.

Lederer (1964) has shown that direct condensation of some fatty acids can and does occur. In experiments using live diphtheria bacilli, it was found that $1-C^{14}$ palmitic acid yielded radioactive corynomycolic acid which was labelled exclusively on carbon atoms 1 and 3. This demonstrated the direct condensation of 2 molecules of palmitic acid to the C_{32} corynomycolic acid.

Structure of Corynomycolic Acid



The Effect of Various Disinfectants on the
Lipolytic Activity of Certain Bacteria

It is known that many bacteria produce lipolytic enzymes capable of breaking down a variety of lipid substrates. We were interested in finding out if sub-lethal concentrations of phenolic disinfectants would destroy the lipolytic activity of certain bacteria known to possess lipases.

Test Organisms

The test organisms used for this experiment were:

Staphylococcus aureus F.D.A. 209

Serratia marcescens

Pseudomonas fluorescens O.A.C. 99

Arthrobacter nov.sp.

All the test organisms were cultured in T.C.S. broth at 37°C for 48 hours before testing for lipase activity.

Medium Employed to Demonstrate Lipolytic Activity

Fat plates were prepared which contained the simple triglyceride tributyrin (3%) in nutrient agar.

Disinfectants Employed

The following disinfectants and the concentration used follow:

Para-chloro-meta-cresol	450 $\mu\text{gm}/\text{ml}$
Phenol	45 $\mu\text{gm}/\text{ml}$
6-chlorothymol	200 $\mu\text{gm}/\text{ml}$
2,4,6-trichlorophenol	200 $\mu\text{gm}/\text{ml}$

Method

A standard 4 mm loopful of broth suspension of test organism was streaked on fat agar plates and incubated at 37°C for 5 days. Evidence of fat hydrolysis is indicated by greenish-blue coloration of fat globules under and around the bacterial growth, when the plates are flooded with a saturated copper sulfate solution for 10 - 15 minutes.

To study the effect of disinfectants on lipolytic activity, 0.2 ml of the test organism suspended in sterile phosphate buffer, was added to 5 ml of disinfectant solution. The concentration of disinfectant used was such that a 3 hour exposure of the test organism would not be lethal to the test organisms. After 3 hours a 4 mm loopful of the test suspension was streaked on a fat agar plate, incubated for 5 days and stained with copper sulfate solution.

Results

The results of this experiment indicated that all 4 of the test bacteria contained lipases capable of hydrolyzing the simple triglyceride tributyrin.

Our results indicate that sub-lethal concentrations of phenolic disinfectants have no effect on the lipolytic activity of the organisms tested. However, it may be possible to devise a test system which is more quantitative and less difficult to interpret

than the gross examination of plates.

Discussion

Alford and Pierce (1963) obtained results which show the wide variability that may occur in lipase production by the same organism grown in different media. The failure of Pseudomonas fluorescens to produce appreciable lipase in their synthetic medium, as well as its production of more lipase on a casein digest than on peptone, is added evidence that a single medium is insufficient for the determination of lipolytic activity by different bacteria. This they say is particularly true if attempts are being made to differentiate degrees of lipolytic activity. Since Pseudomonas fluorescens is similar to Pseudomonas fragi in its pattern of lipolysis, the differences in nutrient requirements for lipase synthesis probably are not caused by a basic difference in the two enzymes, but rather a difference in the synthetic pathways.

Alford, Pierce and Suggs (1964) have shown that there are at least 3 types of extracellular lipases. Lipases of the first type, obtained from several microorganisms, attack primarily the 1-position of the triglyceride in a manner similar to that of pancreatic lipase. Some fatty acid preference was also observed. The ability of the lipolytic enzymes from Staphylococcus aureus and Aspergillus flavus to attack the 2-position as well as the 1-position suggested another type of activity. The third type of lipase was from Geotrichum candidum and is unusual in that it has a high degree of specificity for unsaturated fatty acids; the position of the fatty acid in the molecule has only a secondary effect.

Our tests indicated that bacteria, when subjected to sub-lethal concentrations of disinfectants, were not affected in so far as lipolytic ability is concerned. However, we feel that direct plating of the pre-exposed bacteria to fat agar was not the best test to demonstrate any changes since this test is not quantitative and in some instances the plates were difficult to interpret.

AMINO ACIDSExamination of the Amino Acid Pool of *Pseudomonas* Grown in
Trypticase Soy Broth and Mineral Salts - Mineral Oil Medium

In our attempt to explain the poor growth of Pseudomonas fluorescens in mineral salts - light liquid petrolatum medium, we first looked for differences in the lipid composition of this organism when grown in 2 different media. As an additional test, examinations of the amino acid pools of Pseudomonas grown in trypticase soy broth and mineral salts - light liquid petrolatum were made.

Culture

Pseudomonas fluorescens O.A.C. 99 was grown in T.C.S. broth for 24 hours at 37°C. A culture of the same organism, which had been maintained for 4 weeks on light liquid petrolatum - mineral salts medium, was grown in the latter medium for 5 days at 37°C with constant aeration.

Extraction of Amino Acid Pool

The cells from both cultures were recovered by centrifugation and the pellet washed twice with distilled water. The cells were then suspended in 3 ml of distilled water and the amino acids extracted by boiling for 5 - 10 minutes in a hot water bath. This mixture was then centrifuged and the supernatant collected for qualitation.

Chromatography

Two - Dimensional Paper Chromatography of the Amino Acid Pool

Five inch squares of Blue Ribbon #589 filter paper were used.

Solvents:

Solvent I

tert-butyl alcohol	695
Water	295
Formic acid	10

Solvent II (Rockland and Underwood, 1954)

Crystallized phenol	775
Deionized water	215
Concentration ammonium hydroxide	1.0/94 ml

A stock solution of phenol and water was prepared by melting the crystallized phenol at 60°C and adding deionized water. The stock solution can be stored at room temperature in a stoppered brown glass bottle. Just prior to use as a chromatographic solvent, 94 ml of the stock solution was mixed with 1.0 ml of concentrated NH₄OH. This solvent requires about 5 hours to ascend 16 cm (at room temperature).

Detection of Amino Acids

The filter paper strips were sprayed with 0.2% ninhydrin in acetone and heated at 100°C for 5 minutes to develop the color.

Amino Acid Standards

The following amino acid standards were prepared in deionized water:

L - methionine

L - alanine

L - aspartic acid

L - glutamic acid

L - serine

Eagle MEM 71.059 (Rhodes and Van Rooyen, 1962)

L - tyrosine

L - glycine

L - arginine

Method

The procedure and solvent systems for two-dimensional filter paper chromatography were very similar to those outlined by Rockland and Underwood (1954). Five inch squares of filter paper were spotted about 3/4 inch from the bottom and left-hand corner of the paper via a small bore capillary tube. The filter papers were mounted on glass rods with cellulose tape and suspended in American Medical Museum jars containing enough solvent to allow the paper to dip in about 1/4 inch. The solvent was allowed to migrate to within 1 inch of the upper end of the paper, which for Solvent I took 7 hours. After drying overnight the chromatograms were trimmed along the solvent front and remounted so that the direction of migration was at right angle to the initial migration and inserted in the museum jar containing the same amount of Solvent II as for Solvent I. The time required for the second solvent system was 5 hours.

The papers were then dried at room temperature and amino acids detected using ninhydrin. Faster drying could be achieved by first washing the papers with acetone to remove large amounts of the phenol.

Using two-dimensional paper chromatography 13 spots were obtained on chromatograms prepared from the standard mixture of 13 amino acids. When the amino acid pools from T.C.S. broth grown

Pseudomonas fluorescens O.A.C. 99 and mineral salts - light liquid petrolatum grown Pseudomonas were analyzed by two-dimensional filter paper chromatography, 10 amino acids were detected for the organisms grown in the complex medium (T.C.S.) and only 5 were detected for cells grown in the hydrocarbon medium. No attempt was made to identify the individual amino acids present in the amino acid pools.

Discussion

Two-dimensional filter paper chromatography was utilized in the study of the amino acid pools of Pseudomonas fluorescens O.A.C. 99 which had been grown in 2 different media.

The method involving two-dimensional filter paper chromatography was found to yield 10 amino acids in the pool of trypticase soy broth grown organisms and 5 amino acids in the pool of mineral salts - light liquid petrolatum grown organisms.

It may be assumed from this difference in amino acid pool content, that Pseudomonas fluorescens can only synthesize completely, 5 amino acids from the carbon and nitrogen substrates available in the simple medium. Any others it needs for synthetic processes must be supplied in the medium or synthesized by conversion of those which it can synthesize. This may be one of the reasons why this organism does not grow well in such a simple medium as our mineral salts - light liquid petrolatum.

CHAPTER IV

Identification and Characterization

of a New Species of the Genus

Arthrobacter

CHAPTER IVIntroduction

In the winter of 1963 we observed, in a special culturing apparatus for studying liquid petrolatum degradation, the growth of an organism which had not been placed in the apparatus originally. Our attention was attracted because of the rapidity with which this organism could break down light liquid petrolatum with the development of a visible white scum at the hydrocarbon mineral - salts interface. Since the various species of bacteria we were currently studying, with respect to this property, were unable to accomplish such a marked breakdown, the bacterium was isolated and further investigated.

From the morphological and physiological properties of this organism it was concluded that the bacterium is a hitherto undescribed species of the genus Arthrobacter.

HISTORY OF THE GENUS ARTHROBACTER

In 1928, Conn described under the name of Bacterium globiforme an organism which appeared as Gram-negative short rods in 24 hour agar slant cultures but Gram-positive cocci after the culture was 3 to 4 days old. This organism grew abundantly in good soils but poorly in soils of low productivity.

Taylor and Lockheed in 1937 also reported that there exists in fertile soils, a type of organism having the unusual physiological property of changing from a rod form into a coccoid form and was not necessary for the good growth of plants, it was more abundant in good soils than in less productive soils. This was in agreement with the findings of Conn (1928). It was also shown that the relation of the organism to soil fertility was based mainly on the

available nitrogen fraction present.

Further studies on Bacterium globiforme by Taylor (1938) showed that in many instances the organism still appears as rods even after 5 days. Prolonged incubation was necessary for confirmation of bacterial type. Taylor examined 90 soil samples from various localities in Canada and found Bacterium globiforme in 89. It was seen that in general, numbers tended to be high in more fertile soil, although Lacombe soils were a notable exception. In 2 soil samples whose pH was lower than 5.0, Bacterium globiforme was not only restricted but also were a smaller percentage of the total bacterial population.

To sum up his work, Taylor pointed out that this group of organisms are as abundant in a soil situated on the rim of the Arctic circle as in soils on the Atlantic and Pacific coasts of Southern Canada. It is present in garden, prairie, orchard, marsh and mixed cropped soils, whether acid, neutral or alkaline, fertile or infertile.

Thus, in general, counts of Bacterium globiforme in soils do not provide any more information about the soil than can be obtained from a total plate count except at pH below 5.0, then this organism is severely repressed.

Taylor also studied the morphology of this organism in artificial media and found the following:

- 1) morphology is profoundly affected by time, temperature of incubation and the nature of the substrate.
- 2) on beef-peptone agar, young cultures are consistently short rods which vary considerably in length and tend

to be slightly curved and tend to clump. A few coccoid cells are commonly present.

- 3) At temperatures above 28°C, large distended rods with bulbous extremities, which break into cocci are prevalent. As the culture ages the rods turn into cocci by a process which is virtually a swelling and fragmentation of the rod. This swelling is much more obvious in some strains than in others.
- 4) Time taken to attain the coccoid state varies considerably 48 hours to 120~~0~~ more days for the appearance of an appreciable number of cocci.
- 5) On solid synthetic media, young cultures invariably exhibit rod morphology.

The morphology is more extreme in liquid medium. In old cultures the organism is present as rather large cocci, together with an assortment of pleomorphic rods.

Taylor (1938) found strains that could survive subzero temperatures. It was ascertained that the organisms were not so tolerant of heat as of cold. Ranges of growth temperature varied considerably and though 25°C approximates the optimal growth temperature on nutrient agar plates, most strains produce some growth at 33°C. The Conn strain was the only one found to grow well at 37°C.

Out of 28 cultures incubated at 28°C for 5 days, 14 showed no growth at pH 4.0, 6 showed some growth and 8 grew well. All grew well at pH 6.0, 7.0 and pH 8.0.

Conn and Dimmick (1947) have studied many groups of soil bacteria which are similar in morphology to Mycobacterium and Corynebacterium. They found that many of these bacteria should be grouped under the genus Arthrobacter because of striking morphological differences between these organisms and the Corynebacterium.

One of the most recent reports of the isolation and identification of a new species of Arthrobacter was by Schippers-Lammertse et. al. In 1963 these investigators isolated a bacterium which produced a bluish colony on peptone-glucose agar. The blue pigment was not restricted to crystals in the colonies as the agar also turned blue. This organism appears to be different than Arthrobacter atrocyoneus which also produces a blue pigment indigoidine reported by Kuhn and Starr in 1960.

Experimental

Effect of Temperature on the Growth of Arthrobacter nov.sp.*

To determine the optimal growth conditions for the new species of Arthrobacter, 1 ml of a 20 hour nutrient broth culture of the test organism was used as an inoculum for 100 ml of fresh nutrient broth.

The organisms were grown at 3 different temperatures on an Eberbach Water-Bath Shaker. Optical density readings of the culture were read at 0.5 hour intervals in a Spectronic 20 photometer at a wavelength of 600 m μ .

The optimal temperature for growth of Arthrobacter nov.sp. in nutrient broth was found to be between 25°C and 30°C. The highest titre was found at 25°C.

* nov.sp. - an abbreviation for new species.

This organism is capable of growing both aerobically and anaerobically and is therefore a facultative anaerobe.

The results of this experiment are shown in TABLE XXXVIII and GRAPH XXII.

TABLE XXXVIII

Effect of Temperature on the Growth of Arthrobacter
nov.sp. as measured by Optical Density

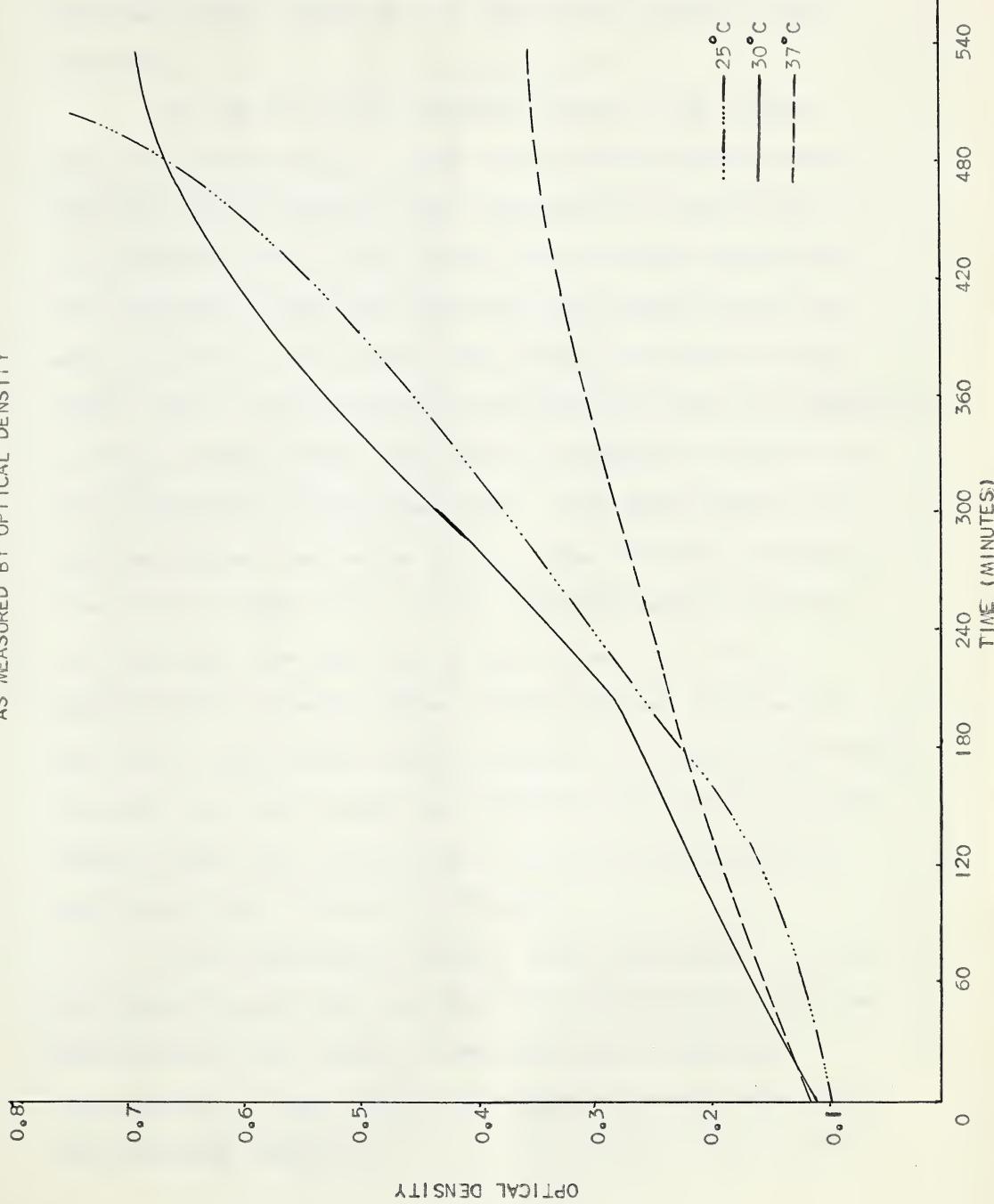
Time (minutes)	Temperature		
	25°C	30°C	37°C
0	0.095	0.10	0.11
30	-	-	-
60	0.13	0.16	0.15
90	0.13	0.18	0.17
120	0.15	0.215	0.185
150	-	-	-
180	0.22	0.25	0.215
210	0.25	0.28	0.23
240	0.27	0.32	0.245
270	-	0.365	0.26
300	0.37	0.42	0.27
330	0.41	0.49	0.28
360	0.46	0.505	0.30
390	0.50	0.57	0.33
420	0.52	0.58	0.33
450	0.60	0.60	0.33
480	0.67	-	0.34
510	0.75	-	-
540	-	0.69	0.36

- indicates no reading taken at this time.

Morphology

The bacterium shows a pronounced pleomorphism, and all morphological characteristics of the genus Arthrobacter could be observed. Growth studies performed on this organism grown on nutrient agar plates, indicated that there is a distinct morphological pattern during the growth cycle. This was established by inoculating a culture of this organism to nutrient agar plates

AS MEASURED BY OPTICAL DENSITY



and at various intervals the culture was examined under the microscope. For photomicrographs, smears were made on glass slides at selected intervals of time during the growth period and these were stained with Gram's stain. PLATES II to X represent the results of this experiment.

At time "0", which represents a smear of the organism grown for 14 days on N.A.P., there are only coccoid forms present (PLATE II). This culture was then transferred to a fresh N.A.P. and incubated at 30°C. After 8 hours the cocci have changed into long rods some of which show transverse septa delineating the new small cells later to be formed. Some of the long bacillary forms exhibit areas of swelling usually toward the end of the cell (PLATE III). At 12 hours (PLATE IV) the long rods have changed into shorter rods with the formation of many cross-walls. At 24 hours (PLATE V) the rods have thickened and become shorter. Some "V" forms are present. After 36 hours (PLATE VI) the culture is predominantly very short, thick rods with a few long bacilli still present. By 45 hours (PLATE VII) the culture is almost entirely composed of chains of cocci with a very few short bacilli present. At 72 hours (PLATE VIII) the culture has almost completely reverted to cocci. Even in 14 days cultures (PLATE IX) it is possible to locate an occasional short bacillus but their occurrence is infrequent.

This organism can be said to stain Gram-variable. In the early phase of growth there are distinctly positive forms and a few negative cells. The cystite or coccoidal form is invariably Gram-positive. In some cultures Gram-negative bacteria with positive granules appear (PLATE IX).

It was noted that in stock cultures, which had been stored at 6°C, (see PLATE X), swellings within the bacillary forms occurred much more frequently and were more pronounced than in cells grown at 30°C (PLATE III).

Growth Cycle of Arthrobacter nov.sp.;
Gram's Stain; 1500X

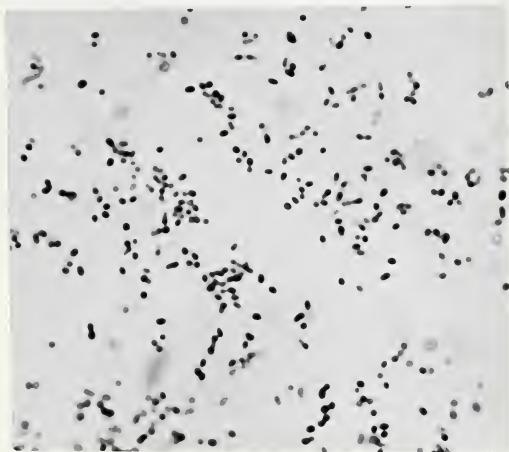


PLATE II "0" hours. Coccoid forms



PLATE III 8 hours. Long rods with areas of swelling.



PLATE IV 12 hours. Short rods with many cross walls.



PLATE V 24 hours. Short thick rods. Some "V" forms present.

Cont.

Growth Cycle of Arthrobacter nov.sp.;
Gram's Stain; 1500X

PLATE VI 36 hours. Short, thick rods with a few long bacilli.

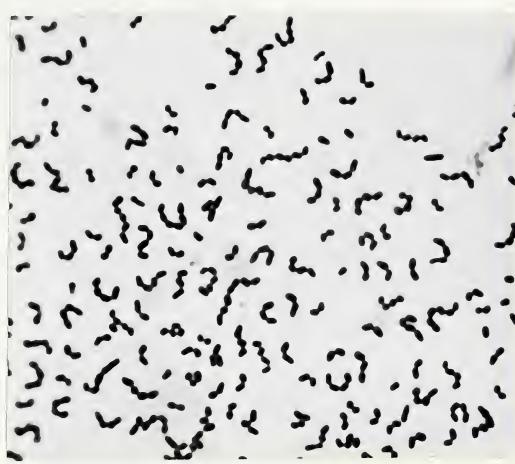


PLATE VII 45 hours. Chains of cocci with a few short bacilli present.

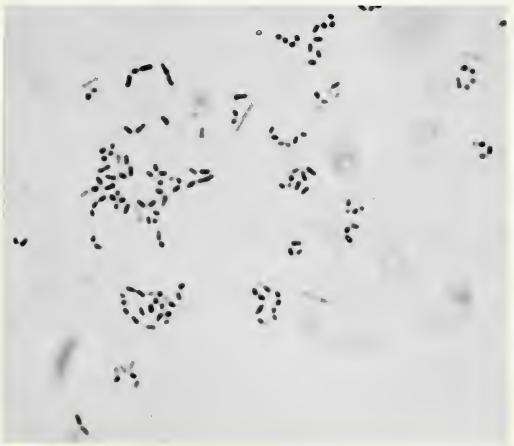


PLATE VIII 72 hours. Predominantly cocci.

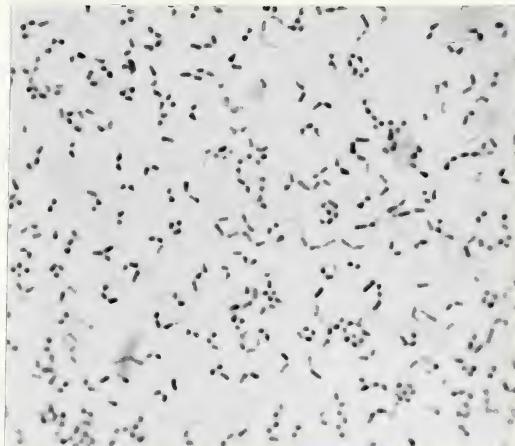


PLATE IX 14 days. Predominantly cocci with an occasional bacillus.

Cont.

Growth Cycle of Arthrobacter nov.sp.;
Gram's Stain; 1500X

PLATE X 23 day old culture which has been stored at 6°C. Frequent and pronounced areas of swelling.

Cultural and Physiological CharacteristicsGelatine Slab

There was no gelatine liquification even after 3 weeks of incubation at 30°C.

Growth on Nutrient Agar and Trypticase Soy Agar (30°C)

When Arthrobacter nov.sp. was grown on N.A.P. there was abundant growth of colonies which were creamy white, circular, smooth, convex, shiny, 1 - 2 mm in diameter, entire edge, butyrous consistency.

On trypticase soy agar the colonies were quite different to those on nutrient agar. The color of these colonies was pinkish-brown and possessed a more matte surface. The color became more intense as the culture aged. The colonies were much more distinct on T.C.S. than N.A.P.

Agar Slant

The growth on nutrient agar slants and trypticase soy agar slants was filiform with characteristics as listed above.

Indole Production

The test organisms were grown in peptone solution for 48 hours at 30°C. The culture solution was then tested for indole by the Erlich's test in which 1 ml of paradimethylaminobenzaldehyde (2 gm/100 ml of 95% ethyl alcohol) was added to the culture, then concentrated HCl was introduced a drop at a time, until a red zone appeared.

The red color should be soluble in chloroform.

The results of this test were negative indicating that no indole was produced.

Litmus Milk (Difco-B107)

No change was evident in the litmus milk after 4 weeks of incubation at 30°C.

Hydrogen Sulfide Production (Kligler Iron Agar)

There was no hydrogen sulfide produced after incubation for 7 days at 30°C.

Acid and Gas Production From Sugars

The sugar solutions were prepared by adding 1% of the sugar to nutrient broth and also adding 1% Andrades indicator. A Durham tube, filled with medium, was inverted within the medium to indicate the production of gas.

There was little or no acid and gas produced from sucrose, lactose or dextrose after 1 week of incubation at 30°C.

Acetone Production

The production of acetyl-methyl-carbinol was tested for by the Voges-Proskauer test.

The absence of the characteristic rose color indicated that no acetoin was produced.

Starch Hydrolysis

The starch agar and Lugol's iodine solution used for this test were prepared according to Levine (1954). The Arthrobacter was inoculated to starch plates and incubated for 5 days at 30°C. The presence of clear zones around the area of bacterial growth, when stained with Lugol's solution, indicated positive starch hydrolysis.

Nitrate Reduction

The nitrate medium and reagents required for the detection of nitrate reduction were those described by Conn (1949).

The production of a slightly more intense pink color in the test system over the control system when treated with sulfanilic acid in 5N acetic acid and alpha-naphthylamine in 5N acetic acid indicated a slight reduction of nitrate to nitrite.

Urease Activity

Urea broth (Difco, B272) was prepared according to the method described in the Difco Manual 9th Edition.

One loopful of the test organism was inoculated to each tube of urea broth and incubated at 30°C.

The pH and color of the test solution and an uninoculated control were noted at various times.

The results of this test are shown in TABLE XXXIX.

TABLE XXXIX

Urease Activity of Arthrobacter nov.sp.

Time (hours)	Uninoculated Control		Test Mixture	
	pH	Color	pH	Color
0	6.8	yellow	6.8	yellow
12	"	"	no change	
45	"	"	7.0	light pink
72	"	"	7.2	darker pink
120	"	"	8.0	bright pink
11 days	"	"	8.5	bright pink

These results indicate that urea was hydrolyzed.

Catalase Activity

Twenty-four hour nutrient agar slants of Arthrobacter

and uninoculated nutrient agar slants (controls) were flooded with 3 or 4 ml of 3% hydrogen peroxide. The presence of bubbles (liberated oxygen) in the test system and not in the control indicated a strong catalase activity.

Growth in Nutrient Broth

Good growth was obtained in nutrient broth in 2 days with the formation of a fine pellicle. The medium was turbid and contained a heavy, viscid, streaky sediment. Little odor was produced and the pH of the growth medium was raised to 8 after 4 days of incubation.

Growth on Peptone-Glycerol-Potassium chloride Agar

(Lammertse *et al.*, 1963)

The following medium was employed:

Peptone	1%
Glycerol	2%
Potassium chloride	0.6%
Agar	2%
Final pH = 9.0	

Excellent growth was obtained on this medium. The colonies appeared the same as when grown on nutrient agar plates.

Growth on Citrate Agar

Simmon's citrate agar was used to determine if the Arthrobacter could use citrate as the sole source of carbon. The formula for this medium is as follows

	<u>Gm/1</u>
Magnesium sulfate	0.20
Monoammonium phosphate	1.00
Dipotassium phosphate	1.00
Sodium citrate	2.00
Bacto-agar	15.00
Bacto-brom thymol blue	0.08

The medium was dispensed as slants and cultures of Arthrobacter were inoculated by streaking. After incubation for 5 days at 30°C there was very scant growth and no color change in the indicator. Thus the Arthrobacter cannot use citrate as a sole carbon source.

Growth on Nicotine Agar (Sguros, 1955)

Formula for Nicotine Agar

Nicotine	0.4
KH_2PO_4	0.2
KCl	0.5
Yeast extract (Difco)	0.01
MgSO_4	0.0025%
FeSO_4	0.0025%
Agar	1.5%
H_2O	

Sguros tested 7 strains of Arthrobacter oxydans and obtained abundant growth with the production of a deep blue diffusible pigment. The pigment turns reddish or yellow-brown with time. This blue material which was formed during the oxidation of nicotine is practically insoluble in all of the common solvents with the exception of benzyl alcohol.

Our species of Arthrobacter did not grow on this medium even after 7 days of incubation at 30°C.

Growth on Asparagine Agar

The medium of Kohn and Starr (1960) was employed.

The formula for asparagine agar is as follows:

K ₂ HPO ₄	1.0
MgSO ₄ ·7H ₂ O	0.2
CaCl ₂	0.1
FeCl ₃	trace
NaCl	0.1
KNO ₃	0.5
Asparagine	0.5
Mannitol	1.0
Agar	15.0
Distilled H ₂ O q.s.	ad 1000 ml

A 24 hour distilled water washed culture of Arthrobacter was inoculated to the asparagine agar plates and incubated at 30°C for 1 week.

The colonies produced were small, whitish cream in color, shiny, raised with a finely indented edge.

Detection of a Capsule

The Arthrobacter was grown on nutrient agar plates for 4 days. A loopful of organisms were suspended in a drop of India ink and observed at a magnification of 960. The presence of a bright halo around the cells indicated the presence of a capsule.

The morphological and physiological characters indicate clearly that this organism has to be classed with the genus Arthrobacter. The organism described here exhibits a number of properties that clearly distinguish it from the previously named Arthrobacter species.

Hydrocarbon Utilization by a New Species of Arthrobacter

Introduction

According to Stone (1963), there are many bacteria that can attack hydrocarbons besides those that most often survive enrichment culture. There is a need for systematic studies on some of the bacteria that do not always win out in a competitive environment when only one carbon source is present. We know very little about the metabolic capabilities of the Arthrobacter or the Corynebacterium strains to mention two genera.

Obviously the necessity of controlling the growth of organisms in oil-water combinations will be greatly increased if the organisms involved can utilize hydrocarbons. We had observed that the new species of Arthrobacter brought about certain physical changes when grown in hydrocarbon, therefore it seemed desirable to attempt to determine the chemical changes which occurred.

Characterization of a Product Produced by Arthrobacter nov.sp. from Light Liquid Petrolatum

Culture

When the new species of Arthrobacter was grown on a 5 - 10% mineral salts - light liquid petrolatum medium for 14 days at room temperature (22 - 25°C), a thick, viscous white material was produced at the oil-water interface. Isolation and identification of this product was undertaken. The medium used in this study is as follows:

Mineral Salts Medium (Raymond and Davis, 1960)Gm/litre of distilled water

Ammonium Sulfate	1.0
Magnesium Sulfate 7H ₂ O	0.2
Ferrous Sulfate 7H ₂ O	0.005
Monobasic Potassium Phosphate	2.0
Dibasic Sodium Phosphate	3.0
Sodium Carbonate	0.1
Calcium Chloride	0.01
Manganous Sulfate	0.002
Ashed Yeast Extract	0.008

To sterilize this medium without the formation of a precipitate, the phosphates were sterilized apart from the rest of the constituents and added when cool. An alternate method was to sterilize by membrane filtering.

The light liquid petrolatum was added as the medium was being used.

Isolation and Characterization of Polysaccharide

After 14 days of incubation, the remaining hydrocarbon was decanted from the incubation mixture and the bacterial cells removed from the aqueous phase by centrifugation.

A preliminary test on the culture supernatant using the method of Dische (1955) indicated that the viscous material was a polysaccharide of some type.

To 0.5 ml of the unknown solution was added 5.0 ml of sulfuric acid (75% w/v) and 0.2 ml of 1% alcohol-indole solution. This mixture was shaken well and heated for 10 minutes in a boiling water bath. A control, which contained distilled water in place of the test solution, was maintained as a standard. The production of a brown color in the test mixture indicated that the test material was carbohydrate in nature since all carbohydrates, with the exception of amino sugars, yield the brown coloration.

The periodic acid test (Shriner et al., 1960) was also used to demonstrate the presence of carbohydrate material. Periodic acid reagent has a very selective oxidizing action of 1,2 glycols, α -hydroxy aldehydes, α -hydroxy ketones, 1,2-diketones and α -hydroxy acids. The rate of reaction decreases in the order mentioned.

Reagents

1. Periodic Acid Reagent

Paraperiodic acid (H_5IO_6) 0.5 gm

Distilled water 100 ml

2. Silver Nitrate Solution

Silver nitrate 5 gm

Distilled water 100 ml

Two ml of periodic acid reagent were placed in a test tube, and 1 drop of concentrated nitric acid, was added with shaking. Then 1 drop of the unknown solution was added. The mixture was shaken for 10 - 15 seconds, and 1 - 2 drops of aqueous silver nitrate solution added. The instantaneous formation of a white precipitate (silver iodate) indicated that the unknown organic compound had been oxidized by the periodate, which was thereby reduced to iodate. This is a positive test for the presence of a sugar like compound in the unknown solution. The method was tested on known compounds, namely L-fucose, sorbitol and glucose all of which gave positive tests. A negative test was obtained with isopropyl alcohol.

After confirming that the material being identified was a polysaccharide of some type, attempts were made to purify the material.

Isolation and Purification

Precipitation of the polysaccharide was accomplished by adding 2 volumes of 95% ethanol per volume of filtrate. The alcohol-water mixture was shaken and chilled overnight and the precipitate centrifuged out of suspension (the precipitate was white and flocculent). The precipitate was purified by solubilizing at room temperature in 0.1% sodium acetate and then filtered through Whatman number 1 filter paper. The polysaccharide was reprecipitated from the sodium acetate by adding 60% ethanol at 5°C. This procedure was repeated 3 times. The final precipitate was redispersed in distilled water and dialyzed against distilled water for 72 hours. The polysaccharide was reprecipitated by adding ethanol and a small amount of sodium chloride. The precipitate was washed several times with absolute ethanol and then with anhydrous ether (Wang, Steers and Norris, 1963).

The presence of nucleic acid was indicated in the polysaccharide preparation by absorption between 260 μ and 268 μ . Most of the ultra-violet absorbing materials were removed by double passage of the polysaccharide solution through an activated charcoal column. Spectrophotometric readings were made on a Spectronic 505 recording spectrophotometer.

The purified polysaccharide material was hydrolyzed by heating in 2 N sulfuric acid for 4 hours in a boiling water bath (saturated NaCl). The hydrolysate was neutralized with Ba(OH)₂ and the precipitated BaSO₄ was removed by centrifugation (Cohen and Johnstone, 1964) and passage through filter paper.

The end product from the hydrolyzation procedure was ninhydrin and nigrosin negative, indicating freedom from protein contamination.

When the neutralized sample was tested for reducing sugars, by adding 10 drops of sample to 5 ml of Benedict's Reagent and boiling for 5 minutes in a water bath, there was a negative reaction thus the absence of reducing sugars in the polysaccharide was demonstrated.

Chemical Characterization of Polysaccharide

Phosphorus Determination

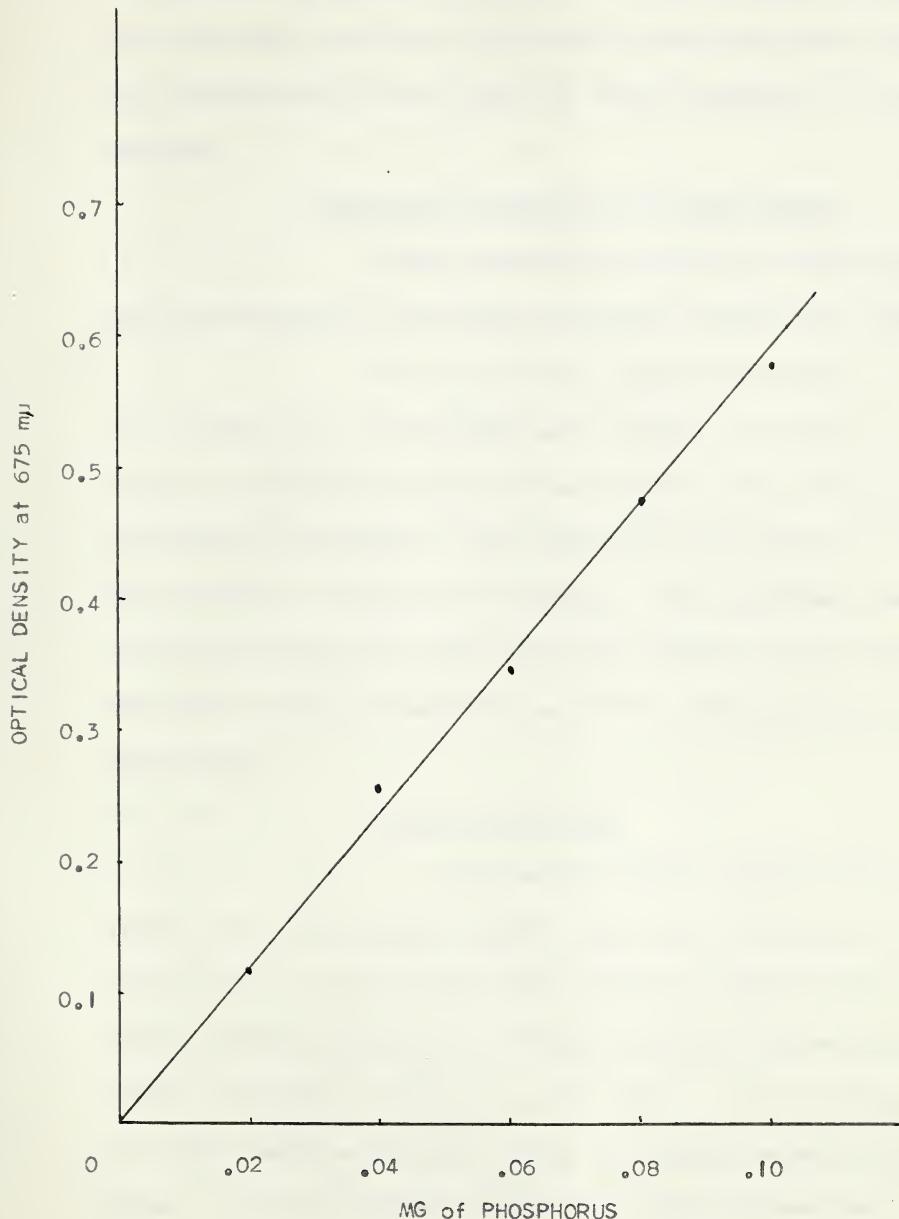
Observations using the Spectronic 505 recording spectrophotometer indicated that small amounts of ultra-violet absorbing materials may still be present in the hydrolyzed polysaccharide sample. A phosphorus determination was thus indicated as a measure of nucleic acids which may be present.

The method used was that of Allen (1940). An appropriate amount of the test material to be examined was placed in a 25 ml volumetric flask and to this was added, 2 ml amidol, 1 ml ammonium molybdate solution, and water to 25 ml. The extinction coefficient was determined 30 minutes later.

A standard curve for phosphorus was prepared from a phosphate solution containing 1 mg of phosphorus per ml (by dissolving 1.0967 gm of dried KH_2PO_4 in distilled water and diluting to 250 ml). Suitable standard solutions for calibration of the photometer were obtained by dilution of the stock solution. Readings were made at 675 μ on a Spectronic 20 photometer.

GRAPH XXIII

STANDARD CURVE FOR PHOSPHORUS DETERMINATIONS (O.D. vs. MG.M.P)



After preparing the standard curve, aqueous solutions of the polysaccharide were tested and readings compared to the standard curve. The results of 3 determinations indicated the presence of about 0.045 mg P per 1 ml of sample. This indicates that there may have been a very small amount of nucleic acid present as a contaminant but not enough to be of consequence in subsequent analyses.

Detection of Methyl Pentose and Hexose

Methyl pentose and hexose were detected by the L-cysteine-sulfuric acid method of Dische and Shettles (1948).

To 1 ml of test solution was added 4.5 ml of a mixture of 1 volume water and 6 volumes of sulfuric acid. During the addition the mixture was cooled in ice. Subsequently the mixture was warmed to room temperature then held in a boiling water bath for 3 minutes or 10 minutes. The tubes were removed from the boiling water bath and cooled quickly in tap water. To the cold solution was added 0.1 ml of 3% aqueous cysteine hydrochloride with shaking.

10 Minute Reaction

With methyl pentose sugars a greenish yellow color develops and remains practically unchanged in intensity for 24 hours. The greenish yellow color is characteristic for methyl pentoses in only the 10 minute reaction whereas pentoses, hexoses, hexuronic acids give a pink color. The absorption maximum for methyl pentoses is about 400 m μ and ranges between 360 m μ and 430 m μ . In the 10 minute reaction the curves of pentoses and hexuronic acids are practically horizontal in this range.

3 Minute Reaction (Primary L-Cysteine-Sulfuric Acid Reaction

In the 3 minute reaction hexoses also show a yellow color. This color decreases rapidly in intensity and except in the case of mannose, changes into another color: green for glucose and ~~fructose~~, blue for galactose and sorbose. In this reaction, all hexoses show absorption between 360 $\text{m}\mu$ - 430 $\text{m}\mu$.

The 10 minute reaction is more specific than the 3 minute reaction but the latter has the advantage of making possible the detection of hexoses in addition to methyl pentoses, in the same sample.

Method

One sample of polysaccharide was subjected to the aforementioned L-cysteine-sulfuric acid test for 10 minutes boiling and a second sample was subjected to the 3 minute boiling water treatment. Both samples were then read in a Spectronic 20 photometer after sitting 5 minutes at room temperature. The sample which had undergone 3 minute boiling was also read after 30 minutes and 18 hours in order to realize color fading which is characteristic of the presence of hexoses.

Results

The results of these determinations are reported in TABLE XL and GRAPH XXIV.

TABLE XL

Determination of the Presence of Methyl Pentose and Hexoses
Using L-Cysteine-Sulfuric Acid Method

Wavelength (m μ)	OPTICAL DENSITY			
	Sample Heated 10 min.	Sample Heated 3 min. (a)*	Sample Heated 3 min. (b)**	Sample Heated 3 min. (c)***
350	0.01	0.035	0.03	0.03
360	0.01	0.045	0.04	0.04
370	0.02	0.06	0.05	0.055
380	0.03	0.085	0.07	0.075
390	0.035	0.115	0.09	0.085
395	-	-	0.10	0.090
400	0.040	0.135	0.11	0.085
405	-	0.14	0.11	-
410	0.035	0.145	0.11	0.065
415	-	-	-	0.065
420	0.03	0.14	0.085	0.040
430	0.02	0.10	0.06	0.025
440	0.01	0.065	0.04	0.02
450	0.005	0.035	0.02	0.02
460	0.00	0.02	0.01	0.015
470	-	-	0.005	0.01
480	-	-	-	-

* (a) read after 5 minutes

** (b) read after 30 minutes

*** (c) read after 18 hours

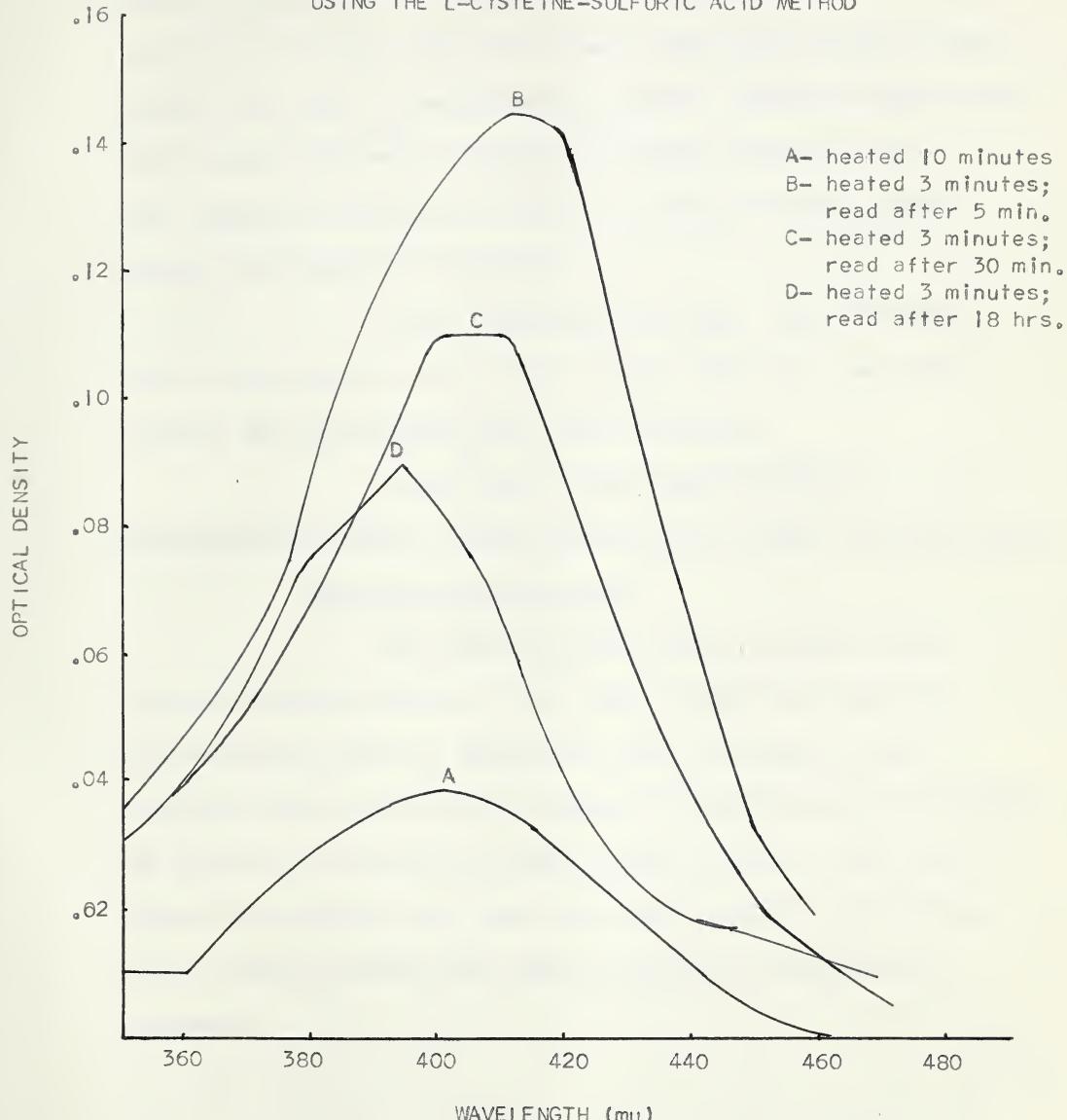
Discussion of Results

The results indicated the presence of a methyl pentose which has a maximum absorption at 400 m μ , and the presence of a hexose. That there was another sugar present was evidenced from the fact that the sample which was heated for 3 minutes decreased in color intensity with time and also showed a shift in maximum absorption point. This shift was from 415 m μ at 5 minutes, to 405 m μ after 30 minutes and 395 m μ after 18 hours.

GRAPH XXIV

DETERMINATION OF THE PRESENCE OF METHYL PENTOSE AND HEXOSE

USING THE L-CYSTEINE-SULFURIC ACID METHOD



Dische et al. (1949) used the adsorption increment between two wavelengths in the primary L-cysteine-sulfuric acid procedure, as a test for hexoses.

This procedure consists of determining the density of a solution at two wavelengths at which the extinction coefficients of other sugars are equal. Thus D_{380} and D_{415} in this reaction are equal for methylpentoses and for the other sugars which show a uniform intensity of absorption between 380 and 430 $\mu\mu$. $D_{415} - D_{380}$ is, therefore zero for all classes of sugars except hexoses, for which it is positive.

In our experiments the $D_{415} - D_{380}$ (3 minute reaction) was positive in both the solution which was read after 5 minutes and the solution read after 30 minutes.

It may thus be said that the unknown polysaccharide contains a methyl pentose and at least one hexose sugar.

Test for Neuraminic Acid

The method of Barry, Abbott and Tsai (1962) was used. Measured samples of the test solution were placed in 18 x 150 mm test tubes to which were added a solution of 30% aluminium sulfate and Ehrlich's Reagent (p-dimethylaminobenzaldehyde). The tubes were covered with aluminium foil, heated to 100°C for 30 minutes in a boiling water bath then cooled rapidly in cold water. Optical density readings were made at 530 $\mu\mu$ in a Spectronic 20 photometer.

Results

There was no difference in absorption between the control tubes, which contained no sample, and the test solutions. Therefore neuraminic acid was not present in the unknown sample.

Test for Hexuronic Acids by the Carbazole Reaction

The carbazole reaction of Dische (1947) was employed in testing for hexuronic acids where a sample of the unknown solution was mixed (with cooling) with concentrated H_2SO_4 . This mixture was heated in a boiling water bath for 20 minutes, cooled and a 0.1% alcoholic solution of carbazole was added with shaking.

Results

The test solution did not yield the typical pink coloration with an absorption maximum at 530 $\mu\mu$ thus no uronic acids were present.

When 3.8 ml of water were added to the test systems, a weak violet color, which turned an intense blue after sitting a few days, developed. This was indicative of the presence of sugars other than uronic acids.

The control solution of glucuronic acid gave a positive test.

Chromatographic Characterization of Polysaccharide

In all phases of the chromatographic investigation into sugar components of the polysaccharide Whatman No.1 chromatography paper was used with the ascending method. All chromatograms were run at 30°C.

Solvent Systems

The solvent systems employed were:

1. Ethyl Acetate-Pyridine-Water (Smith, 1960)

Ethyl acetate	120 ml
Pyridine	50 ml
Water	40 ml

2. Iso-Propanol-Water (Smith, 1960)

Iso-propanol	160 ml
Water	40 ml

Both solvent systems resolved the standard sugars into discrete spots. The disadvantage of Solvent System I was it's obnoxious smell.

Location Reagents

The location reagents employed were:

1. Aniline-Diphenylamine Reagent

Aniline, 1% (1 ml) plus	
diphenylamine, 1% in acetone ... 10 vol.	
Phosphoric acid, 85% ... 1 vol.	

Most sugars react to yield green, blue or brown colors. Sugars such as sorbitol and mannitol do not react.

2. Periodate Reagent

a. Periodic acid (H_5IO_6) 2.28 gm in

100 ml H_2O	1 vol.
Acetone	19 vol.

b. Benzidine, 184 mgm, in 0.6 ml acetic acid.

Add H_2O , 4.4 ml, and acetone 95 ml.

The paper was dipped in reagent "a" and allowed to stand 3 - 4 minutes then dipped in reagent "b". White spots appeared on a blue background which slowly fades. Sucrose,trehalose and glycosylamines are negative.

Standard Sugar Solutions

All solutions were prepared in 10% isopropanol, pentose and hexose sugars in 0.5% solution and sugar alcohols and disaccharides in 0.7% solution except that deoxyribose and rhamnose are comparatively insensitive to location reagents at these concentrations and need to be 2 or 3 times as concentrated. These concentrations were found satisfactory for one-way chromatograms when one loopful was applied at the chromatogram origin.

The standard sugar solutions prepared were:

1. mixture of xylose, fructose, glucose, sucrose, lactose and glucuronic acid
2. mixture of glucose and galactose
3. mixture of glucose and galactose
4. mixture of raffinose and melibiose
5. L-fucose (1.5%)
6. mannitol
7. sorbitol

Method

One loopful of each of the standard sugar solutions was spotted parallel to the unknown sample on 9" x 22" chromatography paper. When the spots had dried, the papers were placed in a glass chromatography tank and allowed to ascend for 18 - 20 hours with Solvent I and about 40 hours with Solvent II, at 30°C. The papers were removed, dried in a fume hood and developed with the location reagents.

Results

Both solvent systems gave good separation of the standard sugars. Two components were detected in the unknown sample but no positive identification could be made. Thus the characterization of the unknown polysaccharide at present is still limited to the knowledge that one methyl pentose and at least one hexose are present.

The Selection of Appropriate Disinfectants for

Oil-Water Combinations Contaminated with *Arthrobacter*

Recently an increasing number of papers have been published concerning the metabolic activities of the genus Arthrobacter and many new species have been described. (Loos, Roberts and Alexander, 1965; Gasdorf, Benedict et al., 1965; Sieburth, 1964; Gunner, 1964; Thoma and Whitney, 1964).

The biological oxidation of petrolatum phenolic waste waters has been reviewed by Ross and Sheppard (1956). These investigators found that certain bacteria exhibited 99.9% efficiency in the removal of phenol during 24 hours for phenol concentrations up to 300 p.p.m. and that certain pure strains can survive concentrations of phenol up to 1600 p.p.m. (0.16%) for short periods (one day) without serious detrimental effects and was able to oxidize phenol with better than 99% efficiency for initial concentrations up to 450 p.p.m. Prolonged exposure to concentrations above 500 p.p.m.

however, resulted in a marked decrease in efficiency.

Therefore it seemed desirable to determine whether or not Arthrobacter species, which we have shown grow profusely and bring about both physical and chemical alterations in hydrocarbon, are capable of utilizing phenol or phenolic compounds since these compounds naturally suggest themselves as disinfectants for oil or oil-water mixtures.

The Utilization of Phenol by Arthrobacter

To study the ability of the new species of Arthrobacter and Arthrobacter polychromogenes (Lammertse et al., 1963) to utilize phenol, 300 p.p.m. of phenol was added to a mineral salts medium and the test organism added. The flasks were completely wrapped in aluminum foil to minimize photo-oxidation of the phenol and after 3 days incubation, 400 μ gm of glucose was added. Incubation of the mixture was for another 7 days on a mechanical shaker at room temperature at which time a loopful of the culture was inoculated to nutrient agar plates to insure that no contamination had taken place.

The contents of the culture vessels were filtered through a membrane filter (0.45 μ) and the supernatant collected and diluted as necessary with distilled water for reading at 270 $\text{m}\mu$ in a Beckman D.U. Spectrophotometer. Values obtained were compared to standards which did not have organisms added. Optical density values were converted to concentration values on the standard curve prepared previously for phenol.

Results

TABLE XLI indicates the decrease in phenol concentration after 10 days incubation at room temperature.

TABLE XLIPhenol Utilization by Arthrobacter polychromogenesand a New Species of Arthrobacter

<u>Test Organism</u>	<u>Concentration of Control</u>	<u>Concentration after 10 days incubation</u>	<u>% Phenol Utilized</u>
<u>Arthrobacter polychromogenes</u>	285 p.p.m.	60 p.p.m.	78.9%
<u>Arthrobacter nov. sp.</u>	290 p.p.m.	245 p.p.m.	15.5%

Discussion

Both Arthrobacter polychromogenes and the new species of Arthrobacter were capable of growing in the presence of 300 p.p.m. of phenol. Arthrobacter polychromogenes was found to utilize phenol better than the new species. As a consequence of these findings it became necessary to determine whether or not the growth of Arthrobacter in an oil-water system, could be controlled by some other phenolic disinfectant.

The Use of 6-chlorothymol as a Disinfectant for Arthrobacter nov. sp. in an Oil-Water System

Previous experiments have shown that at a 1:6000 aqueous solution of 6-chlorothymol will inhibit this organism in less than 20 minutes but a 1:7000 solution requires nearly 2 hours in order to inhibit.

This test was done by placing the disinfectant in the aqueous phase of a 10% hydrocarbon - mineral salts and later by placing the disinfectant in the hydrocarbon phase alone. Concentrations of the disinfectant were made relative to the total volume of the test system, that is 50 ml. A control system contained no inhibitor.

Incubation of the system was for 10 days at room temperature. Results were indicated as positive or negative on the basis of visible growth and development of a white scum at the oil-water interface.

Results

The results of this experiment are shown in TABLE XLII and XLIII.

TABLE XLII

The Effect of Various Concentrations of 6-chlorothymol When in the Hydrocarbon Phase of a 10% Oil to Water Mixture

<u>Concentration (w/v)</u>	<u>Results</u>
Control (no disinfectant)	+
1:200	0
1:400	0
1:500	0
1:800	0
1:1000	0
1:2000	0
1:5000	0
1:6000	0
1:8000	+
1:10,000	+
1:15,000	+

TABLE XLIII

The Effect of Various Concentrations of 6-chlorothymol When in the Aqueous Phase of a 10% Oil to Water Mixture

<u>Concentration (w/v)</u>	<u>Results</u>
Control	+
1:6000	+
1:8000	+
1:10,000	+
1:15,000	+

When 6-chlorothymol is in the hydrocarbon phase, the concentration required to inhibit growth of the Arthrobacter is at least 1:6000 whereas if the disinfectant is in the aqueous phase, a 1:6000 concentration does not inhibit the test organism.

Discussion

These results can be explained on the basis of partitioning since we have shown that 6-chlorothymol, when in 10% hydrocarbon, migrates into an aqueous phase to an extent of 0.65% whereas migration from water to 10% hydrocarbon is about 92%. Thus the concentration of disinfectant in both phases was sub-lethal in the situation where disinfectant was initially placed in the aqueous phase. However when the disinfectant was initially in the oil phase, the concentration obtained was sub-lethal in the aqueous bottom but lethal in the hydrocarbon phase.

Assuming that the initial concentration of disinfectant placed in the hydrocarbon phase was 1:6000 and allowing 10 days for migration to occur, the calculated concentration of disinfectant in hydrocarbon would have been 1:6040 whereas if a 1:6000 concentration of 6-chlorothymol was initially placed in the aqueous phase, the concentration in the hydrocarbon phase after allowing 10 days for migration would have been 1:6520, thus explaining why the Arthrobacter grew in one system where a 1:6000 concentration of 6-chlorothymol was used and not in the other system.

SUMMARY

In order to study some of the problems in the control of bacteria in oil and water systems it was necessary to devise methods for suspending bacteria in oil, in the absence of water, and for detecting viable cells in oil. Lyophilization was used to free the test organisms from water and a new procedure involving migration was found to be the best method for detecting viable cells in an oil system. The membrane filter technique and the reduction of tetrazolium salts were not too satisfactory for the latter purpose.

Our spectrophotometric studies on the dynamics of disinfectants when in oil and water systems indicate that time, temperature and oil to water ratio have an effect on the rate of partitioning of disinfectants in such systems. The rate and amount of partitioning is dependent on the particular disinfectant being studied.

In oil-water systems which contained disinfectant in the aqueous phase and which were shaken for 20 hours at 22° - 25°C. it was found that the greatest migration occurred with 6-chlorothymol (89.5%), then para-chloro-meta-cresol (52.9%), 2,4,6-trichlorophenol (40%), and the least amount with phenol (9%). (See TABLE XIX). When the same study was performed on stationary mixtures the results were nearly the same for 6-chlorothymol (92.1%), less for para-chloro-meta-cresol (16.7%) and phenol (4%), and more for 2,4,6-trichlorophenol (70.8%). When the concentration of oil was increased from 10% to 25% the amount of disinfectant which migrated also increased (see TABLE XX).

When the four test disinfectants were dissolved in hydrocarbon and their migration into aqueous phases measured (TABLE XXI) it was seen that the disinfectants which had exhibited the strongest affinity for the hydrocarbon phase when dissolved in water, exhibited the least amount of migration from oil to water when dissolved in oil.

We were able to follow the migration of disinfectant from one phase to another phase. The time required for maximum migration varied from system to system, but the greater part of the migration usually had occurred within 8 hours. Slight fluctuations in the concentrations of the test solutions after this time indicated the dynamic state of the disinfectant between the two phases.

From the data shown on GRAPHS XIX, XX, and XXI it was shown that the rate of migration of disinfectants from aqueous solutions into hydrocarbon phases is influenced by temperatures. The migration of the 3 disinfectants which were tested was most rapid at the higher temperature (37°C) employed, and slowest at the lowest temperature (6°C). However, the final result is very similar for all temperatures studied.

We were able to correlate the migration of disinfectant from an aqueous solution and the loss of disinfecting ability of a particular system. The bactericidal ability of 6-chlorothymol was markedly reduced by an 8 hour exposure of the disinfectant solution to light liquid petrolatum. This loss of disinfecting ability is related to the 92% migration of 6-chlorothymol from an aqueous to hydrocarbon phase. Our experiments with 2,4,6-trichlorophenol yielded similar results whereas experiments with para-chloro-meta-cresol do not.

Only 16.7% of the para-chloro-meta-cresol migrated in stationary mixtures and no significant decrease in disinfecting ability resulted.

A study on the amino acids and lipids of Pseudomonas fluorescens O.A.C. 99 was done in an attempt to find an explanation for the more profuse growth of this organism in a complex medium such as trypticase soy as compared to growth in the simple light liquid petrolatum - mineral salts medium.

It was found that cells which were grown in trypticase soy broth contained 10 amino acids in the amino acid pool and 11.7% dry weight of extractable lipid whereas cells grown in light liquid petrolatum - mineral salts medium contained only 5 amino acids and 14.1% extractable lipid (an increase of 2.41% extractable lipid).

Our results concerning the effect of disinfectants on the lipolytic activity of certain bacteria indicate that sub-lethal concentrations of phenolic disinfectants have no effect on this lipolytic activity. However, a test system should be devised which is more quantitative and less difficult to interpret than the gross examination of plates.

A new species of the genus Arthrobacter was isolated which has the ability to assimilate at least certain components of light liquid petrolatum and produce a substance shown to be extra-cellular polysaccharide.

Morphological characteristics and some metabolic capabilities of this organism are described.

The ability of the organism to utilize light liquid petrolatum, and to grow in the presence of phenol, emphasize the control problems encountered when such organisms contaminate hydrocarbon reservoirs.

RECOMMENDATIONS

From our studies it is apparent that there are many problems associated with the control of bacteria in oil-water systems.

Solution of some of these problems necessitates:

- a) the use of water soluble disinfectants (microorganisms can survive and multiply in the aqueous phase even though lethal concentrations of disinfectant may exist in the hydrocarbon phase). In addition in the laboratory an oil soluble disinfectant may be effective when a sole carbon source is provided, however under field conditions there would probably be alternate carbon sources available in the aqueous bottom permitting unhindered growth of the organism.
- b) testing the disinfectant under conditions which resemble field conditions as closely as possible.
- c) consideration of the effect of disinfectant partitioning and the effect of oil to water ratio, time, temperature and type of hydrocarbon on such partitioning.
- d) testing a variety of disinfectants (it has been shown that certain microorganisms can utilize substances which we commonly consider to be disinfectants).
- e) determination of the type of organism contaminating the oil-water mixture (we have shown that certain bacteria can not only survive in hydrocarbons but can actually utilize the hydrocarbon as an energy source resulting in chemical and physical alteration of the hydrocarbon).

In addition it is obvious that:

- a) the disinfectant selected must not have a deleterious effect on the material being disinfected.
- b) the cost of the disinfection procedure must be weighed against the amount or severity of damage caused by the presence of bacteria in the system.

Appendix I*

The Rate of Kill of Staphylococcus aureus F.D.A. 209
 By Various Disinfectants in Aqueous Solution

Phenol

Disinfectant Concentration	Time in Contact (minutes)					
	5	10	15	20	25	30
1:50	0	0	0	0	0	0
1:60	0	0	0	0	0	0
1:70	0	0	0	0	0	0
1:80	0	0	0	0	0	0
1:100	+	0	0	0	0	0
1:120	+	+	+	0	0	0

Benzalkonium Chloride

Disinfectant Concentration	Time in Contact (minutes)					
	5	10	15	20	25	30
1:1,000	0	0	0	0	0	0
1:10,000	0	0	0	0	0	0
1:20,000	0	0	0	0	0	0
1:25,000	0	0	0	0	0	0
1:30,000	+	0	0	0	0	0
1:40,000	+	+	0	0	0	0
1:50,000	+	+	0	0	0	0
1:100,000	+	+	+	+	+	+

4-chloro-3-methyl-phenol (para-chloro-meta-cresol)

Disinfectant Concentration	Time in Contact (minutes)					
	5	10	15	20	25	30
1:300	0	0	0	0	0	0
1:500	0	0	0	0	0	0
1:600	0	0	0	0	0	0
1:1,000	0	0	0	0	0	0
1:1,500	+	0	0	0	0	0
1:2,000	+	+	0	0	0	0

Polyvinylpyrrolidone-Iodine
(B.D.H.-Betadine - 1% available iodine)

Disinfectant Concentration (P.P.M. I ₂)	Time in Contact (minutes)					
	5	10	15	20	25	30
1,000	0	0	0	0	0	0
500	0	0	0	0	0	0
400	0	0	0	0	0	0
300	0	0	0	0	0	0
200	+	0	0	0	0	0
100	+	+	0	0	0	0
50	+	+	0	0	0	0
25	+	+	+	0	0	0

6-chlorothymol

Disinfectant Concentration	Time in Contact (minutes)						
	5	10	15	20	30	60	90
1:5,000	0	0	0	0	0	0	0
1:8,000	+	+	0	0	0	0	0
1:10,000	+	+	+	+	+	0	0

The Rate of Kill of *Pseudomonas fluorescens* O.A.C. 99
By 6-chlorothymol

Disinfectant Concentration	Time in Contact (minutes)						
	10	20	30	60	90	120	240
1:5,000	+	+	+	+	+	+	+
1:6,000	+	+	+	+	+	+	+
1:8,000	+	+	+	+	+	+	+
1:10,000	+	+	+	+	+	+	+
1:15,000	+	+	+	+	+	+	+
1:20,000	+	+	+	+	+	+	+

The Rate of Kill of Arthrobacter nov.sp.

By an Aqueous Solution of 6-chlorothymol

Disinfectant Concentration	5	10	15	20	30	60	90	120
1:5,000	0	0	0	0	0	0	0	0
1:6,000	+	+	+	0	0	0	0	0
1:7,000	+	+	+	+	+	+	+	0
1:8,000	+	+	+	+	+	+	+	+
1:10,000	+	+	+	+	+	+	+	+
1:15,000	+	+	+	+	+	+	+	+

- * a) incubation time of the disinfectant exposed bacteria was 72 hours
- b) each experiment was done in triplicate
- c) + indicates growth
- indicates no growth
+ indicates very scant growth

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